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Studies in lactic acid fermentation

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BY

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PART II—III

Part II.

"A STUDY OF THE CHARACTERS OF THE STREPTOCOCCI OF DAIRY LACTIC ACID FERMENTATIONS, WITH SPECIAL REFERENCE TO THE PRESENT STATUS OF THE SO-CALLED STREPTOCOCCUS LACTICUS GROUP."

Cooperation of Mr. Roy C. Avery.

A part of the study to be reported in the following pages was carried on in conjunction with Mr. R. C. Avery. With his kind permission, I have included that work in Part II of this thesis. By the incorporation of this work, it has been possible to strengthen data obtained independently by additional and similar data on a larger number of strains of lactic streptococci.

I wish to take this occasion not only to acknowledge the cooperation of Mr. Avery in that part of the work in which he was actually associated, but also to express my appreciation of the extension of his interest to those parts of the investigation in which he was not an active associate.

Part II.

A STUDY OF THE CHARACTERS OF THE STREPTOCOCCI OF DAIRY LACTIC ACID FERMENTATIONS, WITH SPECIAL REFERENCE TO THE PRESENT STATUS OF THE SO-CALLED *STREPTOCOCCUS LACTICUS* GROUP.

INTRODUCTION

The Streptococcus lacticus group originally proposed by Kruse in 1903 had almost unlimited boundaries and would include practically any nonpathogenic milk souring streptococcus. Today, the group is interpreted in much the same way by many authors. However, notwithstanding the fact that the so-called lactic group may be a most inclusive collection, there is a possibility that the present treatment is somewhat too indefinite to furnish a basis for the answer of a number of questions of biological and economic importance.

Its present status conditions the interpretation of the relation of the lactic type to the various other types recognized in propposed systems of streptococcal grouping. A more complete knowledge of the lactic group would aid most materially in the intelligent interpretation of the significance of streptococci in milk and milk products, and also should precede the assignment of the biological agency of important chemical changes in agricultural products. The establishment of the distribution and source of lactic streptococci and the question of whether this type is also responsible for the lactic acid fermentation of plant products must await the assignment of more definite boundary lines to the so-called Streptococcus lacticus group.

The present indefinite status of the lactic group is in no small part due to the reasons which have complicated the status of practically every group of streptococci. "Various authors in attempting to name or classify streptococci have fixed their attention upon different characters as criteria". (Brown, 1919)

For that reason, many of the frequent comparisons of Streptococcus lacticus with Streptococcus pyogenes could be interpreted only after an interpretation of the authors' conceptions of the meaning of the two terms.

The work of Sherman and Albus in the comparison of "pyogenic" strains presumably of udder origin with lactic strains obtained from soured commercial milk, was a distinct step in advance. The study of the streptococci of cheese by Evans (1918) furnishes still better evidence of the value of a cumulative characterization of the lactic group. By employing a number of characters, she was able to distinguish the true lactic from other types of streptococci which would be labeled Streptococcus lacticus by the usual, casual treatment.

Sherman and Albus proposed the following characteristics to distinguish the lactic type from the so-called Streptococcus pyogenes type; reduction of dyes, inability to ferment sucrose, ability to grow at 10° but not at 43° C. Evans (1918) has characterized the Streptococcus lacticus group as follows: characteristic reduction of litmus milk; production of about 0.12 g. of acetic acid per litre of skim milk; carbohydrates fermented in following order of availability: lactose, salicin, mannitol, and sucrose; formation of crystals in milk cultures by a large percentage of strains; decrease in true acidity of yeast peptone broth of initial pH 6.0 by a majority of strains. These and other characters which have been proposed in the differential study of the lactic group will be reviewed in the following pages.

OBJECT OF INVESTIGATION

The present work is presented not as an attempt to characterize or bound the so-called Streptococcus lacticus group, but merely as a report on the characters exhibited by a number of strains which were present in large numbers in fermentation systems in which lactic streptococci are usually the dominant type.

EXPERIMENTAL

I. Selection and Isolation of Strains.

The number of strains studied in this investigation is not large, but special attention was given to their selection to prevent duplication.

The source of the strains is recorded below.

S	Cream
SK	Commercially pasteurized skim milk
G	Milk
C	Cream
W	Commercially pasteurized milk
M	Milk
MAC	Commercial starter
IN	Ice cream
X	Laboratory pasteurized milk
Z	Laboratory pasteurized milk
PD	Commercial starter
I	Milk
2	Commercial starter
3	Commercial starter
4	Milk
5	Oleomargarine
6	Milk
7	Butter
8	Butter
9	Milk

Strains S to PD were isolated in this laboratory with the following relations in mind:

Strains from milk and milk products: At the time of souring, the lactic type of streptococcus has become dominant in the natural flora of milk. For this reason, milk and cream samples were allowed to undergo natural lactic acid fermentation at room temperature. Plates were then poured from high dilutions to obtain the strains predominating in the various samples. The milk and cream were obtained from different producers since there is a possibility of certain strains becoming locally dominant in a

particular dairy. Only one strain was included from each producer or collecting station.

Strains from "Starters": The lactic type of streptococcus is commonly the microbial agent of commercial "starters". Due to the extensive use of these preparations in the controlled fermentation of milk and cream for the manufacture of butter, fermented milk drinks, and certain cheeses, a study of strains from these sources seemed particularly desirable. Strains were obtained by inoculating sterile milk with the "starter" and isolating the strain dominant in the fermented milk culture after incubation at room temperature.

Strains 1 to 9 were furnished as butter "starters" or as strains of lactic organisms, through the kindness of the Dairy and Bacteriology Departments of the following Agricultural Colleges: Vermont, Pennsylvania, Ohio, Michigan (2), Wisconsin, Kentucky, Florida and Oregon. The original sources of these strains are given as furnished by their contributors.*

All strains were replated three times after the original isolation to insure their purity. Stock cultures were maintained by litmus milk cultures, which were placed in the ice-box after 12 hours preliminary incubation at 30° C. Unless noted otherwise, all characters were obtained by use of inocula of one tenth cc. from 12-hour broth cultures, which had been "invigorated" by four successive 12-hour transfers.

For comparative purposes, the following strains representing other types of streptococci were also included in some of the tests.

*We wish to take this occasion to express our appreciation of their courtesy.

Human hemolytic strains: S67, S271, S84, S13, S125, S72, S52, S273, S70, S55, S23. These strains were furnished by Dr. O. T. Avery of the Rockefeller Institute for Medical Research. They represent a typical collection of hemolytic streptococci from human sources. Most of them were isolated from pathological conditions. The actual source of these strains and a further description of their characters are furnished by Avery and Cullen and by Dochez, Avery and Lancefield.

Hemolytic mastitis and udder strains: V1, V2, C53, C57, C59, C67, C69, M26. These strains were also furnished through the kindness of Dr. O. T. Avery. They represent a collection of hemolytic strains isolated from the udders of cows and from cases of mastitis. A further description of these strains is furnished by O. T. Avery and Cullen, by Jones, and by R. C. Avery.

Cheese strain: Strain MH. is included as a representative of the group of hemolytic "cheese" streptococci studied by R. C. Avery.

Sauerkraut strain: Strain K is included as a representative of a small number of strains isolated from sauerkraut in this laboratory.

II. Morphology

Previous reports:

The early descriptions of Leichmann and of Günther and Thierfelder describe the lactic acid organism as short rods with lanceolate or rounded ends, usually appearing in pairs or short

chains. Kruse, Hölling and Heinemann interpreted them as streptocccci. The morphological resemblance of lactic streptocccci to the pneumococcus was pointed out by Kruse, Hölling, and Saito.

Many attempts have been made to use as a differential character the tendency of lactic streptococci to form elongated rod-like cells. Similar attempts have also been made on the basis of length of chains. These distinctions are no longer considered of differential value. Evans (1918) states that Streptococcus lacticus cannot be distinguished by morphology.

Present observations:

Infusion broth cultures of the lactic strains exhibited chains, varying in length from diplococci to those with 12 to 16 cell members. In milk cultures, shorter chains and diplococci predominated.

III. Reduction Phenomena in Litmus Milk.

Previous reports:

Heinemann (1906) in his studies on the relation of the lactic organisms to other streptococci, reported that "litmus milk is decolorized by Bacterium lactis acidi and all streptococci in the same typical manner". "The solid coagulum turns white leaving a pink ring at the top, which gradually extends toward the bottom". Apparently, this statement included the streptococci from pathological conditions.

Esten (1909) observed that litmus was completely reduced by the true lactic organism before coagulation occurred, (this sequence of coagulation and reduction is the opposite to that reported by Heinemann). He considered the reduction phenomenon exhibited in litmus milk cultures to be a valuable differential character for the lactic organism. Rogers and Dahlberg (1912) found that this test served as a means of distinguishing between strains from the saliva and from the udder of cows. Evans (1916) and Sherman and Albus (1918) have used this test to advantage in comparative studies of lactic and udder types. Hart, Hastings, Flint and Evans (1914), and Evans (1918) have found it of value in the differentiation of lactic streptococci from other types of streptococci found in cheese. Broadhurst (1915) did not find the behavior in litmus milk correlated with the origin of streptococci. Although many of the milk strains included in her study must have been true lactics, she does not mention reduction phenomena. Jensen (1919) does not consider the reduction of litmus milk of any value in the differentiation of lactic acid bacteria.

Salter (1921) has reported the behavior of a number of hemolytic human strains in litmus milk. One of the strains reduced the dye. He believed it a valuable character in distinctions between the more common streptococci of milk and pathogenic strains, although some pathogenic strains may not be differentiated by that means. Salter also describes a number of non-pathogenic hemolytic strains from milk which reduce litmus milk and closely resemble the so-called Streptococcus lacticus.

The characteristic behavior of litmus milk cultures of lactic streptococci is in general use among agricultural bacteriologists as a routine character in the determination of the Streptococcus lacticus group.

Present observations:

Procedure: Medium: Sterile skim milk, cdntaining sufficient litmus to give the milk a robin egg blue color after sterilization. All of the strains of the various types were inoculated in this medium. Frequent observations were made to furnish records of sequence of of reduction and coagulation and to avoid failure to observe possible, transient reduction.

Results: All of the so-called lactic strains rapidly and completely reduced the dye; reduction occurred before coagulation. None of the human or bovine cultures exhibited similar pictures, although incomplete reduction occurred with several of the human strains. The cheese hemolytic strain reduces litmus milk in exactly the same manner as do the lactic strains. The strain from sauerkraut gives only faint, if any reduction.

In conjunction with the 10° and 43° temperature tests, observations were made on the reduction pictures given by the lactics at these temperatures. At 43°, the same sequence of reduction was exhibited as at the usual incubation temperatures. At 10° all strains completely reduce the litmus as the first evidence of growth. However, due to the retardation of coagulation and the slow rate of growth, typical pictures are not always evidenced. The pink color, which returns, in many

cases extended to the bottom of the tube before coagulation occurred.

Although litmus milk culture is a valuable routine, preliminary test for lactic streptococci, it can not serve as an independent differential characteristic. The same reduction is also effected by certain members of other groups; it was exhibited by practically all strains of the hemolytic cheese group studied by R. C. Avery.

IV. Final H-Ion Concentration in Glucose Broth.

Previous reports:

Baehr (1910) reported that a larger amount of titratable acid was formed by lactic streptococci than by strains assigned to the Streptococcus pyogenes group. He believed this relation served as an aid in the differentiation of these two groups of streptococci. Similar statements are reported by seferal authors. Such observations are of course entirely dependent upon the various interpretations of the Streptococcus pyogenes group.

The introduction of H-ion concentration measurements have been of little value in the absolute differentiation of the lactic group. However, the pH values reached by the lactic streptococci in glucose broth have served to place them in the "high acid" group of Ayers (1916). This character would also distinguish them from the "human" type of the so-called Streptococcus hemolyticus group (Avery and Cullen), but the final H-ion concentration of the lactic group varies within approximately the same zone as in the case of the "bovine" strains of the hemolytic group.

Present observations:

Procedure: Medium: Standard infusion broth, pH 7.2, containing one per cent glucose. Tests were made by the method described by Avery and Cullen (1919). H-ion concentrations were determined colorimetrically after 48 hours incubation at 37° C.

Results: The final H-ion concentration varied from pH 4.1 to 4.5. The values for the various strains are reported in the tabular summary.

In this medium, the final H-ion concentrations of the lactic cultures, as an independent character, merely places them in the large and heterogenous "high acid" group of Ayers. In the characterization of lactic streptococci, the value of H-ion concentration measurements seems to be that of a preliminary, but primary, differential character.

V. Behavior on Blood Agar.

Previous reports:

The confusion resulting from the differences in emphasis placed upon the various differential characters used in the characterization of streptococci, is especially evident in a review of the literature on the hemolytic ability of the lactic type of streptococci.

Müller (1906) found no difference in the hemolytic action of "pyogenic" streptococci and of streptococci from milk. However, his report cannot be used in the assignment of hemolysis to the lactic streptococci, as in the selection of his strains he probably ruled out most of the common lactic types upon

morphological grounds. Nieter (1907), Baehr (1910), Shippen (1914) report that the lactic streptococci exhibit no hemolysis. Saito (1912) reported that his lactic strains did not always show hemolysis; when present, it was "usually extended, diffuse, but often not complete". Puppel (1912) investigated a large number of strains from milk and compared them with those from human sources. He reports differences in the ability of the strains to hemolyze different kinds of blood. On rabbit's blood a number of the milk strains showed "strong" hemolysis. Puppel reviews the work of a number of authors and points out that, with the exception of Müller, these authorities agreed that the "milk streptococci" show no, or "only traces" of, hemolysis. Streptococcus lacticus is recorded as non-hemolytic on the blood plate, in von Lingelshein's summary (1912).

Ruedeger (1912) reported that Streptococcus lacticus could be distinguished from Streptococcus pyogenes by the greenish discoloration of blood by the lactic colonies. Broadhurst (1915) reported that "green" or"green haze" discoloration was the only change produced on blood plates by twenty strains from milk (some of which probably were lactic streptococci).

Heinemann (1915) claims that after animal passage, two originally non-hemolytic strains of Streptococcus lacticus acquired the ability to hemolyze to some extent.

Davis (1916, 1918) reported that Streptococcus lacticus usually produces a green colorization on blood agar without appreciable hemolysis. He (1918) also reports on certain hemolytic strains from milk which are at least closely related to his non-hemolytic lactic strains. Salter (1921) has

also reported hemolytic strains from milk which seem to agree with the usual characterization of Streptococcus lacticus in all of the characters which he tested.

Present observations:

Procedure: Brown (1919) has emphasized the need of employing standard procedures in the study of the hemolytic action of streptococci on the blood plate. The conditions he advises were maintained in the present investigation.

0.6 cc. of defibrinated rabbit blood was added to tubes containing 12 cc. melted standard infusion agar, pH 7.4. Properly diluted suspensions were prepared from 12-hour broth cultures of each strain; the blood agar was inoculated and shaken; plates were poured and incubated in moist air at 37° C.

Macroscopic and microscopic observations were made at the end of 24 hours. A warm room was used for these observations and plates were returned to the incubator without delay. At the end of 48 hours incubation at 37° C., the plates were refrigerated at 10° C. for 48 hours. Examinations were then made.

Results:

Description of appearances on blood agar.

After incubation at 37° C.: Two strains, X and PD, exhibit the Beta type of hemolysis (Smith and Brown), Colonies were surrounded by a perfectly clear, colorless zone of hemolysis, after 18 to 24 hours incubation at 37° C. Most of the other lactic strains exhibited zones of greenish discoloration varying in extent. After 48 hours incubation, strains Z, 5 and 7

often produced very wide discolored zones around the surface colonies, at times simulating hemolysis unless examined carefully. Two of the lactic strains, W and S K, and the sauerkraut strain, never produced appreciable discolorization of the medium. Differences in behavior on blood agar seem to be exhibited by the surface and deep colonies of some strains.

After refr igeration: The above strains which had produced methemaglobin at 37° C., exhibited more or less clear zones surrounding a distinct inner ring of non-hemolyzed corpuscles next to the colony. These zones varied in area with the different strains.

The photographs shown in the following plates are offered as types of the different appearances observed.

Table I.

Behavior on Blood Agar Plates
After Stated Periods of Incubation and Refrigeration.

Strain	24 hr. at 37° C.		48 hr. at 37° C.		48 hr. at 8° C.	
	Surface	Deep	Surface	Deep	Surface	Deep
S	Indiff.	Indiff.	Methem.	Methem.	R. Hem.	R. Hem.
SK	Indiff.	Indiff.	Indiff.	Indiff.	Indiff.	Indiff.
G.	Methem.	Methem.	Methem.	Methem.	R. Hem.	R. Hem.
C	Indiff.	Indiff.	Methem.	Methem.	R. Hem.	R. Hem.
W.	Indiff.	Indiff.	Indiff.	Indiff.	Indiff.	Indiff.
M	Indiff.	Indiff.	Methem.	Methem.	R. Hem.*	R. Hem.*
MAC	Indiff.	Methem.	Methem.	Methem.	Methem.	Methem.
X	Hem.	Hem.	Hem.	Hem.		
Z	Methem.	Methem.	Methem.	Methem.	R. Hem.	R. Hem.
PD	Hem.	Hem.	Hem.	Hem.		
IN	Indiff.	Indiff.	Indiff.	Methem.	Indiff.	Methem.
1	Indiff.	Indiff.	Indiff.	Methem.	Indiff.	R. Hem.*
2	Methem.	Methem.	Methem.	Methem.	R. Hem.	R. Hem.
3	Methem.	Methem.	Methem.	Methem.	R. Hem.	R. Hem.
4	Indiff.	Indiff.	Indiff.	Methem.	Indiff.	Methem.
5	Methem.	Methem.	Methem.	Methem.	R. Hem.	R. Hem.
6	Indiff.	Indiff.	Methem.	Methem.	R. Hem.	R. Hem.
7	Methem.	Methem.	Methem.	Methem.	R. Hem.	R. Hem.
8	Indiff.	Indiff.	Methem.	Methem.	R. Hem.	R. Hem.
9	Methem.	Methem.	Methem.	Methem.	R. Hem.	R. Hem.
K	Indiff.	Indiff.	Indiff.	Indiff.	Indiff.	Indiff.

Indiff.	Indifferent
Methem.	Methemaglobin Production
Hem.	Hemolysis of the Beta Type (Smith and Brown)
R. Hem.	Clear Zones after Refrigeration (Alpha Type. (Smith and Brown)
*	Clear Zones but Very Narrow
Methem.	Very Wide Discolored Zones

It is evident in the above table that most of the lactic strains are methemaglobin producers. The two strains which exhibit the Beta type of hemolysis, have also been tested for their hemolytic titre in saline solution of washed rabbit blood cells according to the technique given in the U. S. War Manual No. 6. In this test, they produced complete hemolysis as rapidly as the human strains.

Tests of the behavior of all of the strains on blood agar have been made several times during the past year. While quantitative differences were observed in the methemaglobin production of various strains, in no case did a strain show hemolysis at one time and not at another. Tests made in which extract agar was used as the base, gave inconstant and difficultly interpreted results.

VI. Volatile Acid Production

Previous reports:

Lactic acid bacteria of the Streptococcus lacticus type were formerly supposed to produce lactic acid alone as the product of the fermentation of sugars. (Leichmann) It has been shown, however, (Jensen (1904), Evans (1918), Hammer (1919), that small amounts of volatile acid are also produced in milk cultures of lactic streptococci. Hart, Hastings, Flint and Evans (1914), and Evans (1918) have used the relative amount of volatile acid produced in the fermentation of milk as a character of advantage in the differentiation of certain types of streptococci from the true lactic.

The possible advantages of the measurements of the actual products of the fermentation of sugars by streptococci as a means of describing streptococci are apparently unexplored.

The recognition of types of streptococci in cheese by Hart, Hastings, Flint and Evans (1914) and Evans (1918) is an indication of the differences in volatile acid production of different groups important in agricultural products. From the standpoint of the Streptococcus lacticus group as a member of the larger group of lactic acid bacteria, this is perhaps the most important and fundamental character. However, more should be known concerning the substrate of the volatile acid producing reaction before its use as a character in the grouping of lactic acid bacteria.

Present observations:

Procedure: The volatile acid production in skim milk was determined in the case of five lactic strains.

Flasks containing 500 cc. sterile skim milk were inoculated with 10 cc. of 12-hour milk cultures; analyses were made after 10 days incubation at 30° C. To obtain results comparable to those reported by Evans (1918), the same procedure was employed. Volatile acids were freed by addition of dilute phosphoric acid until culture was acid to Congo red. The cultures were distilled with steam until 2,000 cc. of distillate had been collected. The distillate was neutralized with barium hydroxide and evaporated to small volume. The barium salts were decomposed by addition of sulfuric acid. Volume was made up to 110 cc. and the mixture was distilled by the method of Duclaux. As comparative figures were all that was

desired at the time, the results were calculated and reported in terms of 0.1 N acetic acid.

Results: Jensen and Evans believe acetic acid represents at least most of the volatile acid production of lactic streptococci. All of the data obtained in this investigation do not agree with Duclaux's constants for acetic acid, which may be due to experimental error or to errors inherent in the method.

The cultures tested produced between 9.08 and 13.40 cc. 0.1 N acetic acid in 500 cc. skim milk. These strains showed a close agreement in volatile acid production with those studied by Evans (1918); who found about 0.12 g. acetic acid per liter in milk cultures of Streptococcus lacticus.

The results are recorded in the tabular summary. They are not presented as absolute values, but they serve to show that only small amounts of volatile acid are produced in milk cultures of lactic streptococci.

In spite of the unknown value of the relative volatile acid production in the grouping of streptococci, its importance in a consideration of the lactic streptococci cannot be over emphasized. The importance of such products of fermentation in butter and other dairy manufactures in which the lactics play a prominent part, warrants its further investigation.

VII. Influence of Temperature Upon Growth.

Previous reports:

Optimum temperature: Optimum temperature relations for growth should be based upon rates rather than upon final products. Such determinations are difficult to make, and there is considerable confusion between rates and final products in the literature concerning the optimum temperature for growth of the lactic streptococci. Most authorities agree on 30° as the approximate optimum temperature of the lactic organism.

Limiting temperatures: Leichmann (1896) reported that his lactic strain did not change milk in eight days at 9-12° C.; at 12-14° C. it curdled milk in six and one half days. Kruse (1903), Baehr (1910), Shippen (1914), Sherman and Albus (1918), and others have reported that Streptococcus lacticus has a lower minimum temperature than the so-called Streptococcus pyogenes group. Stowell and Hilliard (1912) came to the conclusion that temperature relations offer the most valuable differential character in distinguishing between the usual streptococci from milk and those from human throats.

The maximum temperature has also been used a s a character of the lactic group. Leichmann (1896) observed scanty growth at 42° in milk cultures of his lactic strain; Sherman and Albus (1918) found most of their lactic strains did not grow in milk at 43° C.

Present observations:

Procedure: Temperatures of 10° and 43°, which were used by Sherman and Albus in their study of lactic and "pyogenic"

udder strains, were chosen as test temperatures. 0.1 cc. of an 18-hour broth culture was introduced into 10 cc. of sterile litmus milk. The medium was brought to the test temperatures previous to inoculation, which were maintained during the procedure. Duplicates were run on each temperature series. The 43° series was incubated fifteen days; the 10° series, forty-two days. Two experiments were performed; one in February, 1920, and the other in March, 1921. The results are given in the following table.

For purposes of comparison, the behavior of the human hemolytic and the udder or mastitis hemolytic strains, to a temperature of 10°, are of interest. This was tested as follows: Inocula of 0.3 cc. of 18-hour broth cultures of each strain were introduced into duplicate tubes of glucose infusion broth. Observations of gross appearance were made weekly for seven weeks, after which time a colorimetric comparison of the pH value of the control and of the test cultures were made as a check on the presence or absence of growth.

Table II.

Growth of Different Types of Streptococci at 10° and at 43° C.

Lactic Strains.

Strains Tested	10° C. 1920 and 1921	10° C. Feb. 1920	43° C. March., 1921
S	growth	no growth	no growth
SK	"	" "	" "
G	"	" "	" "
C	"	" "	" "
W	"	" "	" "
M	"	" "	" "
MAC	"	" "	" "
IN	"	" "	growth
Z	"	growth	"
1	"	"	"
2	"	no growth	no growth
3	"	" "	growth
4	"	" "	no growth
5	"	" "	growth
6	"	" "	no growth
7	"	" "	growth
8	"	" "	no growth
9	"	" "	" "

Sauerkraut Strain.

	1921	1921
K	growth	slight growth

Human Hemolytic Strains.

12 strains	no growth	not tested

Hemolytic Mastitis and Udder Strains.

8 strains	no growth	not tested

Hemolytic Cheese Strain.

MH	growth	growth

Results: With the cultures tested, the 43° temperature test would seem to be of very limited value. The reason for the larger number of positive tests after a year's cultivation is difficult to explain.

The 10° C. temperature test would seem to be of value in differentiating certain types of streptococci from the lactic. All of the lactic milk strains grew at 10° C. After six weeks incubation, most of the strains had coagulated in milk. The cultures which had not produced sufficient changes in the milk to cause its coagulation at 10° C., coagulated within five minutes after being immersed in a 37° C.water bath.

None of the hemolytic human and mastitis strains produced any change in the glucose broth. Although all of the broth had reached a pH value of approximately 6.7, no difference in the H-ion concentration of the tests and controls could be detected.

While growth at low temperatures might possibly serve to distinguish the lactic type from most pathogenic strains, it would not serve as a differential character in comparative studies of other streptococci of more facultative temperature requirements. There are probably many types of streptococci possessing at least as low temperature requirements as that exhibited by the true lactic. As an example, many of the hemolytic streptococci from cheese studied by R. C. Avery grow readily at this temperature.

VIII. Ability to Survive Pasteurization.

Previous reports:

The heat resistance of streptococci is an exceedingly important character, although its value as a differential character of the lactics as a group is of limited application. The ability of different types of streptococci to survive the pasteurization of

milk is of great significance from economic and sanitary aspects. In the extensive and valuable literature which has contributed to the establishment of the conditions of the process, differences are evident in the heat resistance of different streptococci. It has been demonstrated that few, if any, pathogenic strains are able to survive thirty minutes heating in milk at 62.8° C. The lactic group vary among themselves in their ability to survive this heating process. Many of them, however, are able to survive in numbers sufficient to control the subsequent microbial changes in pasteurized milk. Salter (1921) has recently shown that in milk at 60° C., a higher thermal death rate is exhibited by hemolytic pathogenic strains than by "milk" strains.

Present observations:

Procedure: In our experiments, tests were simply made of the ability of the different strains to survive the temperature--time conditions of the usual pasteurization process, as in the older "thermal death point" determinations. It seemed that daily observations of the incubated tests would furnish means of distinguishing strains which survived the process in large numbers from those of which only a few cells survived. For the interpretation of the resistance of lactic strains to pasteurization, this is probably all that is necessary.

Tubes containing 10 cc. of sterile litmus milk were immersed in a water bath. When the milk attained the temperature of 62.8° C., 0.1 cc. of 18-hour broth cultures was added to duplicate tubes. Care was taken not to allow any of the culture to touch the sides of the tube during inoculation. Thermometer in control tube registered between 62.5 and 63° C. during the experiment. At the end of the heating period, the tests were plunged into running water at 10° C., and the tubes were then incubated at 35o C. Observations were made daily for one week.

Table III.

Ability to Survive the Pasteurization Process.

Strains killed*		Strains surviving Coagulated 24-60 hr.	Strains surviving Coagulated 84-120 hr.
(Lactic)		(Lactic)	(Lactic)
S	S 32 (Human)	SK	2
G	C67 (Mastitis)	PD	4
C	K (Sauerkraut)	Z	5
M		X	
MAC		1	
IN		Man. (Cheese)	
5			
6			
7			
8			
9			

*Strains not surviving tests made both in 1920 and in 1921 are recorded in this group.

Results: The surviving strains may be arranged in two groups. The first group includes strains which seem to be able to survive pasteurization in large numbers. The second group includes strains which survive the process only in small numbers. It is probable that such lactic strains, if present in raw milk would be outgrown by more resistant strains after the pasteurization process. It is of interest to note that both of the hemolytic sour milk strains are included in the more resistant group.

The "human" and "bovine" strains included for comparison did not survive the process. (Salter found certain of his human strains survived 30 minutes at 60° C. in milk tests which had received very large inocula.) The "cheese" hemolytic strain survived the process, which is suggestive of the apparently general, resistant characteristics of the collection of strains of this type.

The value of the survival of this heating process as a differential character of any particular group of streptococci, is probably negligible. Strains vary within the different groups and there are other types of streptococci which may include as large a number of resistant strains as are found in the lactic group. From a practical standpoint, however, the heat resistance of the streptococci of milk assumes considerable moment in the determination of the types of streptococci which will control the subsequent biological changes in pasteurized dairy products, in cheeses which are heated during their manufacture, etc.

IX. Pathogenicity.

Previous reports:

Baehr, Puppel, Saito and Gminder obtained negative results on tests of pathogenicity of Streptococcus lacticus to laboratory

animals. Hölling reported that mice are sometimes killed by injections of Streptococcus lacticus. Heinemann (1907, 1915) has reported an observation of increase of virulence of Streptococcus lacticus after repeated passage through rabbits.

The hemolytic milk strains of Davis (1918) and of Salter (1921) at least closely resemble the so-called Streptococci lacticus. Davis found that most of the strains gave negative results, although two of them seemed to show moderate pathogenic powers for rabbits. In tests made upon rabbits, Salter obtained "entirely negative results" from intravenous injections of his strains. The same results were obtained when mice were used. The effect of animal passage upon the virulence of these litmus milk reducing hemolytic strains, was tested by successive injections of typical strains into six mice. From the results of these experiments, Salter concluded that "it does not seem possible to render a strain of the hemolytic streptococci virulent by passage through mice".

Present work:

Procedure: 0.5 cc. of an 18-hour broth culture of each lactic strain was injected intraperitoneally, into white mice. (These tests were made soon after the cultures had been isolated from their sources, with the exception of those strains which were received from other laboratories).

Results: None of the mice exhibited any deviation from the normal control.

Although the question of virulence is always of first importance in a discussion of the biology of an organism, the value of inoculation of animals as a test of pathogenicity of streptococci is conditioned by many factors. Negative tests may be difficult to accept as final, but (as pointed out by Salter in his report of

similar experiments with hemolytic streptococci from milk), "when a large number of organisms of similar properties give constant results some conclusion may be warranted".

X. Sensitivity to Methylene Blue.

Previous reports:

Sherman and Albus (1918) reported that lactic streptococci reduced methylene blue in milk in a concentration of 0.005 per cent, and that udder strains of the "pyogenic" type failed to reduce the same concentration.

R. C. Avery, in studying the behavior of a large number of hemolytic streptococci from various sources, has shown that milk cultures containing 0.02 per cent concentration of this dye serve as a means of separating hemolytic strains into two more or less well defined groups. The non-hemolytic strains from various sources could not be easily separated upon this basis. A considerable number of udder strains reduced the dye in a concentration four times the strength of that used by Sherman and Albus. This suggests that a division between lactic and udder strains upon this basis is not clearly defined.

Present observations:

Procedure: Milk containing 0.02 per cent of Merck's medicinal methylene blue, received 0.1 cc. inocula of each of the lactic strains.

Results: The lactic strains are comparatively resistant to methylene blue, as complete reduction of the dye was effected by all of the strains of that type.

XI. Fermentation of Carbohydrates.

1. Carbohydrates fermented.

2. Comparative availability of sucrose and lactose to sucrose fermenting lactic strains.

Previous reports:

1. Carbohydrates fermented:

Leichmann (1896) reported the fermentation of lactose, dextrose, maltose, and dextrin by the classical lactic organism.

Later authorities agree on the following fermentation reactions. Dextrose, lactose, and maltose are fermented by all strains; salicin and maltose seem next in order of availability; raffinose is seldom fermented and glycerol and inulin are almost never attacked.

There is considerable dispute over the fermentation of sucrose. Leichmann and Bazarewski (1900) report that it is not attacked. Jensen (1919) would not assign sucrose fermenting streptococci to the Streptococcus lacticus group. Sherman and Albus found but six out of fifty lactic strains fermented sucrose, and believed that failure to produce acid from this test substance was of value in the differentiation of the lactic from the "pyogenic" under types. The percentage of sucrose fermenting strains in their collection is much smaller than in those studied by Evans and other investigators. Jones, who found a number of sucrose fermenters in a collection of lactic strains, places considerable emphasis upon the ability of certain lactic strains to attack that sugar.

2. Comparative availability of sucrose and lactose to sucrose fermenting lactic strains:

It is well known that certain lactic acid bacteria prefer sucrose to lactose as a source of energy, as reports on sucrose

preferring strains are frequent in the literature of the fermentation of plant products. It seems that the relative availability of these two disaccharides in the case of sucrose fermenting lactic streptococci, is at least as important as the mere ability of these strains to form acid from that substrate. This relation has been tested in the second of the following experiments

Present work:

1. Carbohydrates fermented:

Procedure: Evans (1918) has shown that the final pH values reached by lactic streptococci varies with the strain and with different carbohydrates. Hence, determinations of the final H-ion concentrations in the various media did not seem to offer valuable means of characterizing these strains. For this reason, tests were simply made of the ability of the strains to produce sufficient acid to give Andrade indicator a definite magenta color.

Infusion broth, pH 7.2, was used as the base of the test media. This medium fulfilled Holman's requirement, as all strains grew well in it in the absence of a fermentable carbohydrate. One per cent of the test substance and one per cent of Andrade indicator were added to the basic medium.

2. Comparative availability of sucrose and lactose to sucrose fefmenting lactic strains:

The availability of dextrose, lactose and sucrose as sources of energy to the sucrose fermenting lactic strains and to the sauerkraut strain, was tested by a comparison of the rate of acid production exhibited by equal inocula of these strains in fermentation systems differing only in the carbohydrate substrate.

Media: Standard infusion broth, pH 7.2, containing 1.1 per cent of Andrade indicator and of the test sugars. Medium was tubed

in 12 cc. portions in test tubes of uniform bore, and heated for three minutes at 120° C. No differences in the initial pH of the different sugar broths could be detected colorimetrically.

One cc. of 12-hour broth cultures of the test strains was introduced into 100 cc. sterile salt solution. One cc. of the dilution was inoculated into duplicate tubes of each of the test sugar broths. The test media were held at 37° C. throughout the manipulation and were then incubated at this temperature. Observations were made at 15 minute intervals and records made of the time required for the attainment of a distinct pink color. The color of the tests was compared with that of a strip of pink paper.

1. Carbohydrates fermented:

Results: Dextrose, maltose and lactose were fermented by all strains; glycerol was not attacked by any of the strains tested. The results with the other test substances are recorded in the tabular summary.

The strain from sauerkraut fermented the following test substances; glucose, maltose, sucrose, raffinose, lactose and salicin.

With the exception of PD, the lactic strains exhibit the following order of availability of carbohydrates: lactose, salicin, mannitol and sucrose. This is the same order of availability as that given by Evans (1914, 1918) in her descriptions of the lactic group. A larger proportion of sucrose fermenters was found than that reported by Sherman and Albus. The fermentation of sucrose does not seem to be correlated with any other character, as may be seen in the tabular summary. This is also the case with the strains described by Evans (1918). This fact emphasizes the dangers

attending a division of the lactic group upon a single character, such as Jones' suggestion of Streptococccus lacticus I and Streptococcus lacticus II upon the basis of sucrose fermentation.

Table IV.

Comparative Availability of Sucrose and Lactose to Sucrose Fermenting Lactic Strains:

Relative Rate of Acid Production in Various Sugars.

The rate of acid production is compared to that of glucose as unity.

Glucose	Lactose	Sucrose
K	1.00	.22	.92
W	1.00	.94	.94
1	1.00	.75	.56
2	1.00	.80	.62
3	1.00	.71	.55
C	1.00	.82	.53

2. Comparative availability of sucrose and lactose to sucrose fermenting lactic strains.

It is evident in the above table that none of the sucrose fermenting lactic strains exhibited a preference for sucrose. It is probable that the sauerkraut strain is a member of the large group of lactic acid bacteria which are particularly adapted to the fermentation of sucrose. It is believed that the value of the acid fermentation of sucrose as a character of the lactic group is limited, and that its only value would lie in the ruling out of strains which exhibit a striking preference for that substrate.

Differences in the rate of acid production from different carbohydrates have been observed frequently. This had been evident throughout all of our work on the carbohydrate fermentation reported before. In the case of several of the lactic mannitol fermenters, acid production was evident only after four to six days incubation, even with inocula of 0.1 cc. With the sauerkraut strain, the fermentation of lactose and salicin never occurred until after several days incubation. The rate of acid production from these two carbohydrates is strikingly different in the case of the lactic strains, in which group lactose and salicin were both fermented within 24 hours with the inocula used.

It is probable that, in certain cases, great differences in rates of acid production from different carbohydrates represents a distinction between substrates which serve as sources of energy for growth and those which are simply fermentable by enzymes (which may not be elaborated or liberated until later in the history of the culture). It would seem certain that the acid fermentation of salicin and lactose does not serve as a source of energy for the growth of this particular sauerkraut strain.

XII. Coagulation of Milk.

Previous reports:

A few authors have attempted to distinguish lactic streptococci from other streptococci by this character. Others have stated that "pyogenic" streptococci may be distinguished from the lactic type by the time required for coagulation of milk cultures of the two types. Jensen (1919), in fact, describes Streptococcus pyogenes as a type which is unable to curdle milk.

Present observations:

With the large and "invigorated" inocula used throughout our tests, all of the lactic streptococci studied here coagulated milk within 24 to 36 hours.

While it is true that most lactic streptococci curdle milk readily, this characteristic is by no means uncommon among hemolytic "human" and udder strains. Among the twelve hemolytic "human" strains studied in this investigation, seven curdled milk; all but one of the seven hemolytic "udder" strains also exhibited this character. Coagulation of milk is a comparatively frequent occurrence among the various types of streptococci.

Loss of ability of lactic streptococci to coagulate milk has been reported frequently. Several of our lactic strains failed to curdle milk when inocula from old cultures were used. However, no permanent loss of this character was observed. Although in some cases a large number of repeated transfers were required, all strains finally responded to successive sub-cultures, which were incubated at room temperature. It is possible that some lactic strains are temporarily weakened by continued cultivation at 37° C., although we have no experimental evidence definitely supporting this assumption.

XIII. Table of Characteristics of Strains Studied.

Strain.	Litmus milk. Reduction preceding coagulation.	Volatile acids. 0.1 N. acetic acid in 500 cc. skim milk culture.	Behavior on blood agar.	Final pH in glucose infusion broth.	Temperature-growth relations. Growth at 10° C.	Growth at 43° C.	Survival of pasteurization.	Growth in methylene blue. Milk (0.02 per cent). 1:5000 Reduction and coagulation.	Virulence for white mice.	Sucrose preferred to lactose.	Fermentation of carbohydrates. Salicin.	Mannitol.	Sucrose.	Raffinose.	Inulin.	Glycerol.
		cc.														
X	Complete.	(1)	B-hemolysis.	4.2	+	+	+	+	Survival.	-	+	-	-	-	-	-
PD	"	"	"	4.3	+	+	+	+	"	(1)	+	+	+	+	+	-
G	"	11.50	Methemoglobin.	4.2	+	-	-	+	"	-	+	-	-	-	-	-
S	"	13.40	"	4.5	+	-	-	+	"	-	+	+	-	-	-	-
Sk	"	9.08	Indifferent.	4.2	+	+	+	+	"	-	+	+	-	-	-	-
C	"	12.60	Methemoglobin.	4.3	+	-	-	+	"	-	+	+	+	-	-	-
W	"	10.80	Indifferent.	4.3	+	-	-	+	"	-	+	+	+	-	-	-
M	"	(1)	Methemoglobin.	4.5	+	-	-	+	"	-	-	-	-	-	-	-
MAC	"	"	"	4.5	+	-	-	+	"	-	-	-	-	-	-	-
IN	"	"	"	4.2	+	±	-	+	"	-	+	+	-	-	-	-
Z	"	"	"	4.5	+	+	+	+	"	-	+	-	-	-	-	-
1	"	"	"	4.2	+	+	+	+	"	-	+	+	+	-	-	-
2	"	"	"	4.1	+	-	+	+	"	-	+	+	+	-	-	-
3	"	"	"	4.2	+	±	-	+	"	-	+	+	+	-	-	-
4	"	"	"	4.2	+	-	+	+	"	-	+	+	-	-	-	-
5	"	"	"	4.3	+	±	+	+	"	-	+	-	-	-	-	-
6	"	"	"	4.5	+	-	-	+	"	-	+	-	-	-	-	-
7	"	"	"	4.3	+	±	-	+	"	-	+	-	-	-	-	-
8	"	"	"	4.2	+	-	-	+	"	-	+	+	-	-	-	-
9	"	"	"	4.2	+	-	-	+	"	-	+	-	-	-	-	-
(cheese) MAN	"	"	B-hemolysis.	4.1	+	+	+	+	"	(1)	+	+	-	-	-	-
(sauerkraut) K	Not reduced.	"	Indifferent.	4.2	+	+	-	-	(1)	+	+	-	+	+	-	-
(human) S 32	" "	"	B-hemolysis.	5.0	-	(1)	-	-	"	(1)	+	-	+	-	-	-
(mastitis) C 67	" "	"	"	4.4	-	"	-	-	"	"	+	-	+	-	-	-

(1) Not determined.

GENERAL DISCUSSION

All of the collection isolated from sour milk and from fermented dairy products would be included in the so-called Streptococcus lacticus group by the usual, casual treatment. It is shown in the above table, that most of these strains possess the following characteristics in common: reduce litmus in litmus milk before coagulation; coagulate milk; in glucose infusion broth reach final H-ion concentrations more acid than pH 5.0; grow at low temperatures; reduce methylene blue in milk containing 0.02 per cent of the dye; exhibit no pathogenicity to white mice; ferment carbohydrates in the following order of availability; dextrose, lactose, salicin, mannitol and sucrose; do not ferment glycerol. None of the strains fermenting sucrose prefer that substrate to lactose. All cultures tested showed the production of only small amounts of volatile acid in milk culture. Methemoglobin production was the usual behavior on the blood plate.

These results, together with those of Evans (1918) and of Sherman and Albus (1918), suggest that a large number of the streptococci conderned in the lactic acid fermentation of dairy products possess a certain number of characteristics in common. Whether such a collection represents a natural group is another question and it is not the purpose of this paper to propose any definite boundaries to the so-called Streptococcus lacticus group. The possible advantages to be accrued from at least a temporary recognition of certain types as a working basis for their further study have already been suggested.

On the other hand, the assumption and recognition of definite groups of streptococci entail certain disadvantages, depending in no small degree upon the characters chosen as salient boundary marks

of these groups. These disadvantages are evident in Jensen's recent classification of lactic acid producing streptococci, in which a large number of possibly closely related types are separated and defined. Likewise, it seems that Jones' suggestion of Streptococcus lacticus I and Streptococcus lacticus II, not only should await an establishment of the Streptococcus lacticus group itself, but should be based on a more fundamental character, if indeed such a division is desirable at all. For these reasons, it must be admitted that work with a larger number of lactic strains than are reported here, might indeed show that the characters given above for the typical Streptococcus lacticus lead to like disadvantages in attempts to bound the larger lactic group of streptococci.

The value of any system of grouping streptococci is in a large measure dependent upon its usefulness as a working basis for further study of their economic application and of their sanitary significance. From such a standpoint, definite characterization of the lactic group might prove of very limited value to the medical bacteriologist. On the other hand, for the agricultural bacteriologist, more definite characterization would furnish a more intelligent basis of study and seems to be required. Frequently throughout the literature, certain physiological reactions are assigned to the Streptococcus lacticus group, with no attempt to establish any other characteristic of the strains involved, than that of acid coagulation of milk. It is in this connection that the various interpretations of the boundaries of the lactic group assume the greatest moment.

Even in the present incomplete knowledge of streptococcal relationships, the indiscriminate use of definite but meaningless

group names is not desirable. Streptococci actively coagulating milk may not be members of the so-called Streptococcus lacticus group, merely because they were isolated from dairy products. For meagerly described strains from milk, it would seem that more definite terminology than "milk streptococci" is to be questioned.

Two of the strains exhibited the Beta type of hemolysis on the blood plate and actively hemolyzed rabbit blood corpuscles in saline solution. The exhibition of hemolysis by these strains reises the interesting question of whether or not some of the hemolytic streptococci of milk would conform to Evans' more definite characterization of the Streptococcus lacticus group. In the absence of data on the relative amounts of lactic and volatile acid produced in the fermentation of milk, it is impossible to answer this question. It may be seen in the tabular summary, however, that the hemolytic strains agree with all of the other characters of the lactic group. It is possible that these strains, together with those reported by Davis (1918) and by Salter (1921), represent examples of the overlapping of present systems of nomenclature and classification of streptococci.

It is desired to call attention to the source of the two hemolytic strains described in this paper, and to their apparent fitness for the struggle for microbial supremacy in milk and milk products. The existence of such strains suggests that at times hemolytic streptococci may be the predominating type in some samples of milk even during the later periods of its handling. Again, such hemolytic streptococci may be added in large numbers as "starters" in the manufacture of various dairy products. These products, whether pasteurized before or after the inoculation of the "starter", would contain hemolytic streptococci in large numbers. While these strains are probably of no sanitary significance, the

temperature relations of such strains may explain cases in which large numbers of hemolytic streptococci are found in milk and milk products. In the usual method of grouping hemolytic streptococci these strains would be included in the "bovine" type and could be distinguished from hemolytic streptococci of human origin by the method of Avery and Cullen (1919).

SUMMARY

A review of the literature on the lactic group of streptococci is presented, in which emphasis is placed upon the need for more definite information regarding the boundaries of this group.

The lactic strains studied were isolated from sour milk, "starters", and other fermented milk products, as probable sources of the so-called Streptococcus lacticus. These strains have been subjected to a number of tests which have been used by different authors in the description and differentiation of different types of streptococci.

From this study, we have reached the following conclusions:

1. The literature reports that many strains of the type usually dominant in sour milk, possess a number of common physiological characteristics. These may or may not represent a natural group. A summation of characteristics by a large number of workers may serve in the future recognition of the group as a type.

2. At the present time, there is no differential character which can be used as an independent test to distinguish this group. Certain characteristics seem to offer means of differentiating the lactic streptococci from certain other types, but different criteria must be used in different cases.

3. Two strains of nonpathogenic hemolytic streptococci exhibit characters which suggest that hemolytic strains may not only be present in milk or milk products, but may take an active part in the lactic fermentation of dairy products.

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Plate.

TYPES OF BEHAVIOR
EXHIBITED ON BLOOD AGAR BY STREPTOCOCCI FROM SOUR MILK.

(Explanation of the Plate.)

Fig. 1.
Appearance of Blood Agar Colony of Indifferent Strain.

Strain SK. Colony after 48 hours incubation at 37° C.; showing no change of the blood corpuscles surrounding the colony.

Fig. 2.
Usual Appearance of Colony of Methemaglobin Producing Strain.

Strain S. Colony after 24 hours incubation at 37° C.; showing zone of discolored corpuscles surrounding the colony.

Fig. 3.
Appearance of Colony of Methmeglobin Producing Strain After Refrigeration.

Strain S. Colony after 48 hours refrigeration at 10° C., following 48 hours incubation at 37° C.; showing a clear zone surrounding an inner ring of non-hemolyzed but discolored corpuscles next to the colony. The clear zone appears upon refrigeration of blood plates of methemaglobin producing strains after previous incubation at 37° C. This phenomenon is termed the Alpha type of hemolysis by Smith and Brown.

Fig. 4.
Appearance of Blood Agar Colony of Beta-Hemolytic Strain from Sour Milk.

Strain X. Colony after 18 hours incubation at 37° C.; showing a wide clear zone as the result of hemolysis of the blood corpuscles surrounding the colony. This is termed the Beta type of hemolysis by Smith and Brown.

Fig. 5.
Appearance of Blood Agar Colony of Beta-Hemolytic Human Strain.

Strain S32. Hemolytic human strain included for comparison with the hemolytic sour milk strain shown in Fig. 4.

PLATE.

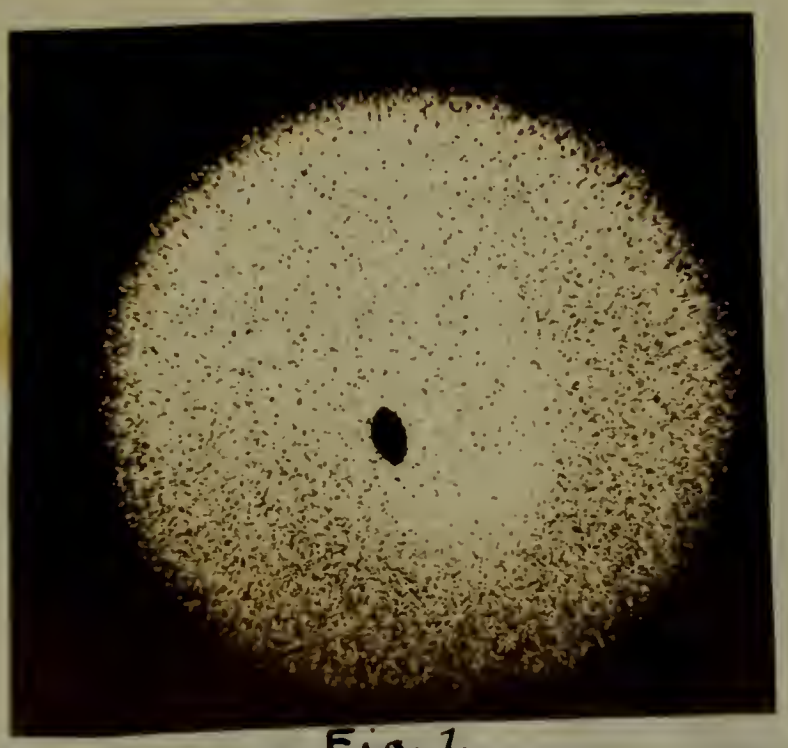

Fig. 1.

Fig. 2.

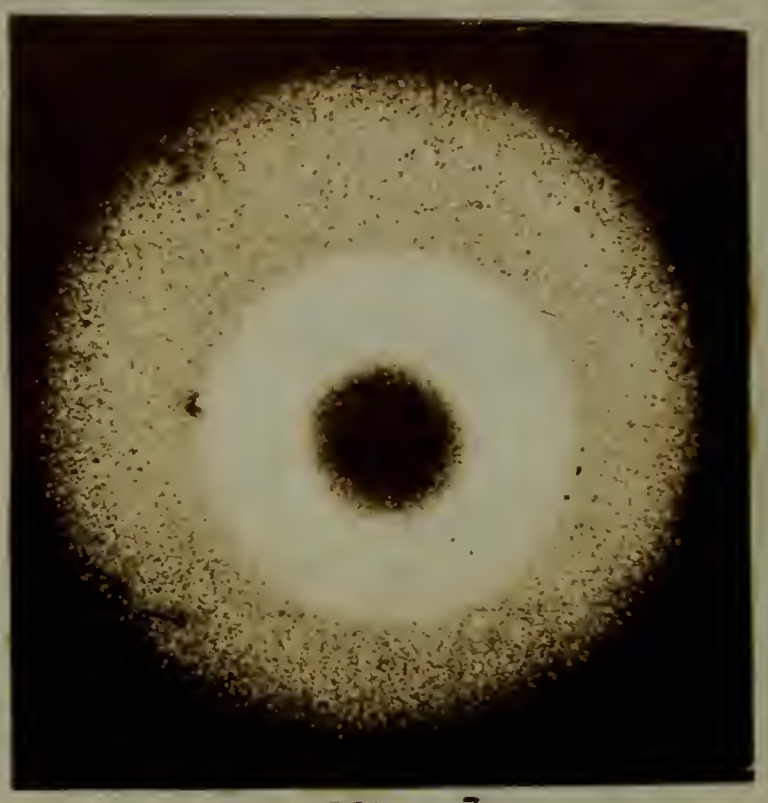

Fig. 3.

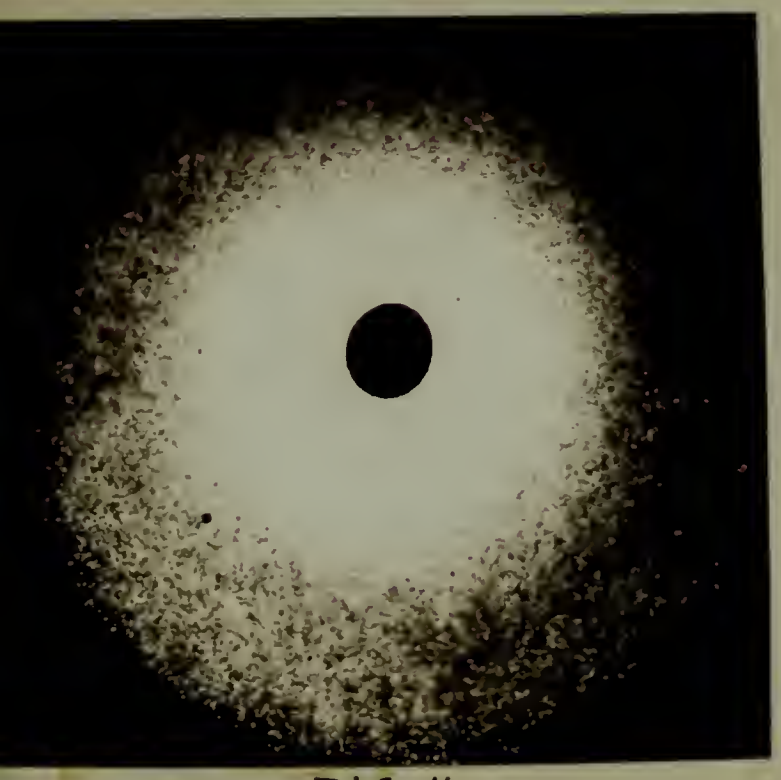

Fig. 4.

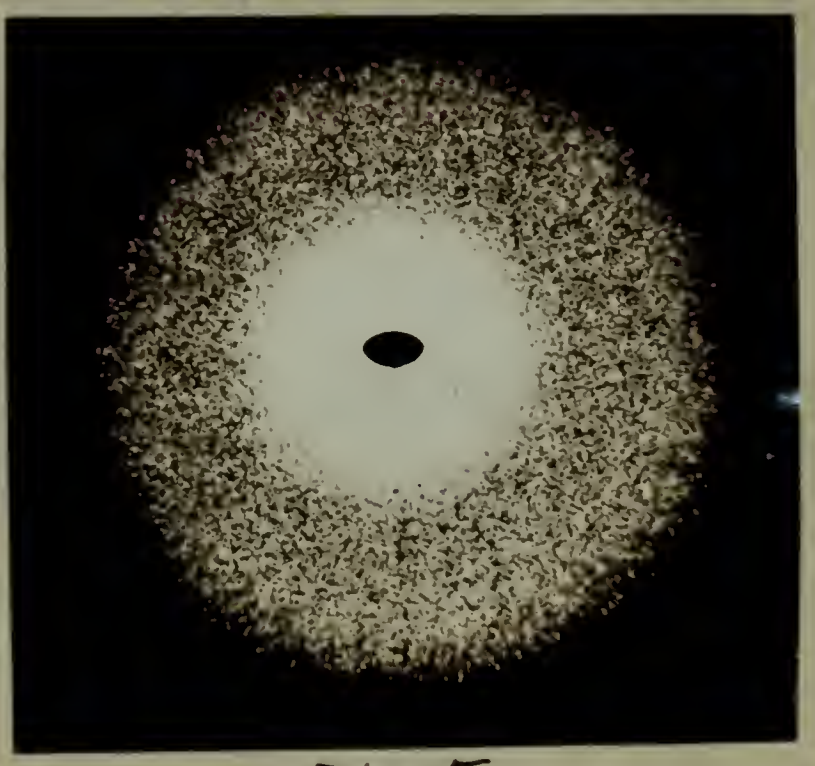

Fig. 5.

PART III.

"A COMPARATIVE STUDY OF THE PEPTOLYTIC ACTIVITY OF DIFFERENT TYPES OF STREPTOCOCCI, WITH SPECIAL REFERENCE TO THE INFLUENCE OF ENVIRONMENTAL CONDITIONS".

Part III.

"A COMPARATIVE STUDY OF THE PEPTOLYTIC ACTIVITY OF DIFFERENT TYPES OF STREPTOCOCCI, WITH SPECIAL REFERENCE TO THE INFLUENCE OF ENVIRONMENTAL CONDITIONS."

INTRODUCTION.

I. Activities of Streptococci in Nature.

II. Significance of Proteolytic Action of Streptococci.

III. Grouping of Streptococci as a Basis of Physiological Study.

IV. Groups Chosen for Study.

INVESTIGATION.

Purposes.

A comparison of the peptolytic activity of different types of streptococci.

A recognition of the significant influence of environmental conditions upon the biochemical activity of microorganisms results in the division of the purposes of the study into the following two sections.

(A.) A comparison of the relative influence of environmental conditions upon the peptolytic activity and other life processes of different types of streptococci.

Here, the study is focused upon the establishment of optimal environmental conditions for the peptolytic action of the different streptococci.

(B) A comparison of the peptolytic activity of different types of streptococci under the foregoing standardized optimal environmental conditions.

General Methods.

I. Chemical Methods.

1. Determination of amino nitrogen.

2. Determination of ammonia nitrogen.

3. Control of volume of tests.

II. Bacteriological Methods.

1. Condition of inocula and purity of tests.

2. Methods used for the approximate comparison of the relative numbers of active cells, or for comparison of the "general growth condition" or vitality of different cultures of the same strain of streptococcus.

Section A.

THE RELATIVE INFLUENCE OF ENVIRONMENTAL CONDITIONS UPON THE LIFE PROCESSES OF DIFFERENT TYPES OF STREPTOCOCCI FOR THE PURPOSE OF STUDYING THEIR PEPTOLYTIC ACTIVITY.

I. Influence of H-ion Concentration upon the Peptolytic Action and Other Life Processes of Different Types of Streptococci.

1. Influence of H-ion concentration upon amino nitrogen increases.

2. Influence of H-ion concentration upon the growth and viability of different types of streptococci.

   a. Relative acid tolerance of the different types.
   b. Influence of H-ion concentration upon the rate of growth and longevity of different types of streptococci.

3. Summary and discussion of the influence of H-ion concentration upon the life processes of the different type strains.

II. Influence of the Stage of Growth of the Culture Upon the Increases in Amino and Ammonia Nitrogen.

1. Experimental.

2. General discussion of the relation of growth stage of the culture to increases in amino and ammonia nitrogen.

   a. Nature of nitrogen distribution in peptone broths.
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III. Influence of Temperature Upon the Peptolytic Action and Upon Other Life Processes of Different Types of Streptococci.

1. Influence of different temperatures upon amino nitrogen increases.

2. Comparison of amino nitrogen increases at 37° and 41° C.

3. Influence of size of inoculum upon amino nitrogen increases at optimum temperature, and at temperatures above the optimum.

4. Influence of temperatures above the optimum upon the final H-ion concentration in glucose broth.

5. Influence of temperature upon the rate of growth, and upon the activity and vitality of different types of streptococci.

   a. Relative rate of growth of the different streptococci at different temperatures.
   b. Comparison of the relative activity and vitality of cultures of the different types of streptococci when incubated at different temperatures.

6. Summary and discussion of the influence of temperature upon the life processes of the different types of streptococci.

IV. Influence of Oxygen Concentration Upon Amino Nitrogen Increases.

V. Optimal Environmental Conditions for the Different Types as Shown by the Foregoing Study.

Section B.

COMPARISON OF THE PEPTOLYTIC ACTIVITY OF DIFFERENT TYPES OF STREPTOCOCCI UNDER THE FOR GOING STANDARDIZED OPTIMAL ENVIRONMENTAL CONDITIONS.

I. Preliminary Statements.

1. Relation to preceding studies.
2. Description of groups and strains studied.

II. Comparison of Amino and of Ammonia Nitrogen Increases Exhibited by Different Members of the Lactic Group, and by Strains of Other Types of Streptococci.

III. Comparison of Amino Nitrogen Increases Effected by Different Types of Streptococci.

IV. General Discussion of the Comparative Peptolytic Activity of Different Types of Streptococci.

INTRODUCTION.

I. Activities of Streptococci in Nature.

Streptococci appear in the most divergent rôles in nature. The lactic group of streptococci are recognized as important agents of lactic acid fermentation. As such, they are employed in the production of butter, certain cheeses, various fermented milks and other dairy products. Again, the activities of certain streptococci are involved in the preparation of many fermented plant food stuffs, as silage, sauerkraut and certain pickles. Some streptococci are frequently associated with severe human pathological conditions. Others are commonly found in the udders of cows and are associated with mastitis; still others appear to be harmless inhabitants of the alimentary and respiratory tracts of man and other animals. Due to the obvious importance of the various types of streptococci, more definite knowledge concerning their metabolism should be obtained.

Knowledge of the physiological properties of the organism is essential to an appreciation of the actual means by which different streptococci produce important changes, whether in milk, in butter, in cheese, or in the human body. It is only by the gradual illumination afforded by cumulative investigations that their fundamental life processes, and the conditions controlling their operations can be interpreted. Such an interpretation must be the foundation of intelligent application of their activities in agriculture, and, likewise, must underlie the

intelligent control of the insidious activities of other kinds of streptococci in the production of disease.

II. Significance of Proteolytic Action of Streptococci.

Among the varied processes involved in microbial activities, those concerned in the nitrogen metabolism of streptococci have received comparatively little attention. Altho', under certain conditions, streptococci are essentially acid forming microorganisms, their proteolytic activities assume importance from many aspects. Following from the importance of proteolytic changes in systems in which streptococci may be the active agents, the significance of this phase of their metabolism ramifies from a more or less common center into processes of moment in agriculture, medicine, and public health.

III. Grouping of Streptococci as a Basis of Physiological Study.

Certain important considerations must precede the active investigation of any of the physiological processes of different members of the streptococcus genus. Before assigning definite functions to certain streptococci, it must be more or less definitely understood to what particular members of the genus, the results of such researches may be projected.

Notwithstanding the probable fallacies of a systematic classification of streptococci, an intelligent pursuit and interpretation of investigations of any of their physiological processes require a certain differentiation between different

streptococci. In spite of the fundamental differences which would seem to exist among microörganisms which function in such different ways as do many of the streptococci, this genus has been unique in its resistance to the usual differential methods of the bacteriologist. For this reason, methods of differentiation of streptococci are usually limited to distinguishing between certain large groups. These groups and their contents have varied with the method of classification and with the purpose of the investigator.

IV. Groups Chosen for Study.

That there are many of these groups must follow from the varied activities of streptococci. Those chosen for study in this investigation represent only four of the most important divisions : (1) the so-called "lactic" group of sour milk, (2) the "human" hemolytic group, (3) the "bovine" hemolytic group, (4) the "cheese" hemolytic group.

The "lactic" type represents the large group of streptococci which function as agents of the lactic fermentation of milk and milk products. The "human" type of hemolytic streptococci represents a large group usually from human sources and frequently associated with pathological conditions. This group is distinguished from other hemolytic streptococci by the lower H-ion concentration reached in glucose broth cultures (Avery and Cullen). The "bovine" type or "high acid" group of hemolytic streptococci probably includes strains from various sources in nature. In this investigation, the "bovine" group of hemolytic streptococci is represented by strains obtained from the udder of cows. Such

strains are frequently associated with mastitis. The "cheese" hemolytic type represents a large group of hemolytic streptococci which seem to be quite commonly present in cheese.

These groups have been established by bacteriologists as convenient bases for further study. They have been proposed by the original authors as a convenient means of treating in a collective manner, certain large collections of strains having a number of common characters. The fact that their members also have a more or less common economic importance lends value to these divisions and furnishes a logical basis for collective treatment.

Comparative studies of these different types of streptococci seem particularly desirable in the face of their obvious economic importance. The importance of the lactic group has been developed in Parts I and II of this thesis. The human type of streptococci is perhaps most important in medicine. However, its close relationship to the lactic streptococci renders comparative studies of the two types an essential step in the gradual development of a future true understanding of the rôle of streptococci in agriculture. Much the same may be said in the case of the bovine or mastitis group of streptococci. The possible importance of the large number of hemolytic strains included in R. C. Avery's cheese group, can not be overlooked in a survey of the probable biological agents of cheese ripening. These four groups are particularly deserving of comparative studies because of the fact that not infrequently all four of these

types of streptococci may be found in one sample of market milk. Here, a knowledge of the conditions controlling the successful operation of their life processes, will furnish some idea of the possibilities of their continued growth in the milk or in products manufactured from milk, - in short, their significance in dairy foods.

INVESTIGATION.

Purposes:

The general purpose of the investigation is a study of the peptolytic activity* of different types of streptococci.

While our principal interest is centered upon the lactic streptococci of dairy lactic fermentations, the peptolytic action of the lactic streptococci is compared with other important types of streptococci which are closely related to the lactics, and which also are of undoubted significance in dairy products.

A comparison of the extent or "activity" of any biochemical process requires a strict definition of the system. This is true in the case of the study of any group of microörganisms; it is especially required in the case of streptococci, which are among all microörganisms perhaps the most responsive to environmental conditions. Hence, a comparative study of the peptolytic activity of different types of streptococci requires a recognition of the important influence which certain factors in the environment may exert upon the action of the peptolytic processes of microörganisms.

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*The term "peptolytic activity" is used in this paper as an expression of the increases in amino nitrogen which follow the bacterial cleavage of the various compounds present in "peptone". The nitrogen of the mixtures of partially hydrolized protein products known commercially as "peptone", is largely in the form of peptide nitrogen. By peptide nitrogen is meant nitrogen found in the peptide linkings, the -CO-NH- groups that link the different amino acids together in peptides, proteins or intermediate products. The cleavage of the compounds included in "peptone" may thus be termed "peptolysis", - a term indicative of the nature of the substances undergoing hydrolysis. The process of "peptolysis" consists in the splitting of the peptide groups, from each of which is generated a carboxyl group and an amino group, as indicated in the following equation (Van Slyke, 1917)

$$RNH\text{-}CO\text{-}R' \longrightarrow RNH_2 + R'COOH.$$

For further discussion of the relations involved see Van Slyke (1917), and pp. of this paper.

With these facts in mind, the purposes of the investigation naturally fall under the following two direct objects -- of sequent significance rather than of coordinate value.

(A) A comparison of the relative influence of environmental conditions upon the peptolytic activity and other life processes of different types of streptococci.

In this section, the influence of environmental conditions is investigated for the purpose of establishing the optimum conditions for the peptolytic action of the different streptococci.
In addition to the establishment of the optimum conditions as an intelligent basis upon which to define the systems in which the comparisons are to be made in Section B, Section A will also present data of independent value.

(B) A comparison of the peptolytic activity of different types of streptcococci under the foregoing standardized optimum environmental conditions.

In this section, the peptolytic action of different streptococci is compared under the optinal environmental conditions which were established in Section A.

General Methods.

I. Chemical Methods.

Measurements of amino nitrogen and of ammonia nitrogen were chosen as the basis of the study of the peptolytic action of the streptococci. The study was limited to the production of these products in meat infusion "peptone" broth, the usual medium of cultivation of these organisms. While the results so obtained may not apply to the action upon intact proteins themselves, or even upon more complex protein derivatives, they at least serve as an index of the action of streptococci upon peptides and other constituents of commercial "peptone".

1. Determinations of amino nitrogen.

Amino nitrogen determinations were made by the nitrous acid method of Van Slyke. The micro apparatus described in 1913 was used with the 5 cc. gas burette described in 1915. Direct determinations were made from 2 cc. samples of the culture.

Conditions and precautions prescribed by the author of the method were observed. In addition, all determinations were made under temperature conditions varying only between 22° and 25° C. Duplicate determinations were made of each test.

Amino nitrogen is expreseed in tables given in the text as mg. NH_2-N per 100 cc.

2. Determinations of ammonia nitrogen.

Ammonia was determined by the method described by Van Slyke and Cullen (1914, 1916). Careful attention was given to the conditions governing the accuracy of the method (Van Slyke and Cullen, 1916).

10 cc. of saturated solution of K_2CO_3 were added to 10 cc. of the sample under analysis. Two drops octyl alcohol were added to prevent foaming and ammonia was aerated into 25 cc. of .02 n HCL. The acid neutralized was determined by titration with 0.02 n NaOH, using sodium alizarin sulphonate as indicator. Ammonia nitrogen is reported in tables in the text as mg. NH_3-N per 100 cc.

3. Control of volume of tests.

All cultures were incubated in moisture saturated incubators; evaporation of tests was controlled by weight.

II. Bacteriological Methods.

1. Condition of inocula, and purity of tests.

Unless stated otherwise, all test cultures received inocula of 0.1 cc. of 12- to 18-hour broth cultures which had been "invigorated" by at least 4 successive 12-hour transfers.

The purity of all test cultures was controlled by microscopic examinations and cultural tests. The latter consisted of subcultures upon infusion agar plates for detection of contaminations, or of milk cultures to distinguish between the type strains themselves.

2. Methods used for the approximate comparison of the relative numbers of active cells, or for comparison of the "general growth condition" or vitality of different cultures of the same strain of streptococcus.

a. The plate method.

In the first of our experiments, the number of viable cells is approximated by the usual plate method. This is the commonly accepted method in general microbiological studies. At best it can present but approximate figures. The errors inherent in the method are particularly evident in the case of long chained

streptococci. Possibly in part due to this fact, the plate method did not prove very satisfactory in the case of tests of the "human" and "bovine" strains. (This difficulty is also reported by Foster (1921)). Moreover, considerable labor is involved in making a determination of a large series by this method, as plates should be poured at least in triplicate to insure any degree of accuracy at all. In case of tests in which the worker has no previous knowledge of the approximate number of cells present in a sample, a still larger number of plates at different dilutions is necessary.

For these reasons, the following method was introduced in the latter part of our work.

b. Principle of method used in part of this investigation.

Streptococci are essentially acid forming organisms. When these organisms are present in systems presenting an easily fermented sugar, they utilize that substrate as the primary source of energy. Upon the introduction of these organisms into glucose broth, their reproduction is dependent upon the energy yielded by the lactic fermentation of the sugar. This reaction yields a product, increases of which are easily detected. Hence, these relations may be regarded as established: (1) the fermentation of glucose is the basic and essential life process of lactic acid producing streptococci; (2) this reaction yields a product whose accumulation can be measured accurately.

It remains to establish the conditions determining the rate of acid production. These relations have been discussed in detail in Part I of this paper ("Theoretical Progress of Lactic Acid Fermentation".) The following is but a brief statement of the

general principles determining the rate of production of the acid product. The acid produced in glucose medi by growth of streptococci may be regarded as dependent upon the multiplication of cells. The miltiplication, of course, is dependent upon the number of cells in the initial inoculum, and will follow in a general way the curve of organic growth, during the earlier period of the fermentation. In this earlier period before inhibiting factors influence the curve of growth, the rate of acid production in a definite and ideal system brought about by a defined fermenting agent is then dependent upon the number of active cells present in the initial inoculum which is introduced into the fermentation system. These relations will hold for a particular strain of streptococci, provided the organisms introduced into the test system are able to begin multiplication at an equal rate.

The conditions influencing the initial phenomena of growth are discussed in the reference given above. However, it may be briefly stated here that, at least in an ideal culture medium, growth will be initiated at an orderly rate, provided the inocula are taken from young cultures which have not been subjected to unfavorable environments.

The principles of this method developed above may be restated as follows: (1) the fermentation of glucose in glucose media is the fundamental, energy obtaining life process involved in the growth of lactic acid producing streptococci; (2) this reaction yields a product whose concentration can be measured accurately; (3) the rate of production of this reaction product is a function of the rate of multiplication of the bacteria introduced, provided tests are limited to the early stages of the reaction in the ideal fermentation systems described.

c. Manipulation of the method.

It is desired to compare either the relative number of active cells, or the "general growth condition" of different cultures of the same strain of streptococcus. A series of cultures of the same strain are to be tested with either of the above objects in mind.

Equal amounts of each culture in the series is introduced into equal amounts of glucose broth. The original inocula represent equal volumes of each test in the series. The glucose broth represents an ideal fermentation system in which the rate of acid production is dependent upon the multiplication of the bacteria introduced. The increase in the concentration of the acid is detected by an indicator present in the system. The time required for the production of acid sufficient to give the indicator a definite color is recorded.

The detailed technique follows: Each culture in the test series is shaken thoroughly to ensure the removal of a representative sample. One cc. of each member of the test series is introduced into 100 cc. of sterile physiological salt solution. These dilutions are shaken thoroughly. (Dilutions of test cultures are employed merely as a more convenient and probably more accurate means of obtaining a small sample of the original test.) One cc. of the dilution is then introduced into duplicate tubes of 12 cc. of glucose infusion broth containing 1.0 per cent Andrade indicator. (The glucose broth is tubed in measured quantities; test tubes of uniform bore are used.) The inoculated glucose broth is shaken thoroughly to distribute the inoculum. These glucose broth test cultures are then incubated at 37° C. in a Wassermann bath. (The glucose broth used in the test is brought to a temperature of 37° before inoculation and is maintained at that temperature throughout the manipulation.)

Observations are made at 15 minute intervals. The glucose broth tests are shaken several times during the incubation period. The time required for the attainment of a distinct pink color of the Andrade indicator is reported in the case of each of the tests. The color of the tests was compared with a strip of pink paper as a means of obtaining an end point of approximately equal value. The advantages and disadvantages of this indicator will be discussed in following paragraphs under the advantages and disadvantages of the method itself.

d. Use of the method.

The uses of any method of comparison are dependent upon its principles. The principles of this method have been stated above. For the convenience of the reader and the interpretation of the comparisons in which it has been employed, they may be festated as follows: Under equal temperature conditions, the time required for the production of a definite concentration of the reaction product is dependent upon the speed of the reaction. The speed of the reaction of lactic fermentation in its earliest period is dependent upon the number of cells and the multiplication of the fermenting agent. The method as a whole then is dependent upon the conditions influencing multiplication. Its uses then are dependent upon these conditions.

The conditions determining multiplication have been given in detail in Part I of this paper. ("Theoretical Progress of Lactic Acid Fermentation") Nothing more will be given here than a statement of the conditions under which the method has been used.

I. Approximate comparison of the numbers of active cells in different cultures of the same streptococcus.

This case requires the strictest definition. Not only must all of the conditions involved in the preceding discussion be observed, but comparisons must also be limited to certain definite series of cultures.

Cell multiplication, and consequently acid production, are dependent upon the ability of the number of cells inoculated to initiate growth. In ideal environments, "lag" and differences in rate of multiplication are dependent upon the vigor of the cells. Hence, the acid production and multiplication of the bacteria will follow the curve of growth only if the cultures are seeded with young cells which have not been subjected to previous unfavorable conditions.

Hence, for reasons given above, the method described can be used for a comparison of numbers of cells in the case of young cultures. Under the conditions prescribed it is believed that

the time required for the production of a definite amount of acid, is a function of the initial number of cells.

This set of conditions was maintained in the study of the influence of different temperatures upon the rate of growth. Here, a series of flasks containing equal volumes of broth were held at different temperatures. Each flask then received equal inocula of the lactic streptococcus. After 12 hours incubation, the relative rate of growth in the different members of the series was compared. Figures were obtained which show the same general relations as those obtained by plate counts made at the same time upon the same series.

Cultures of greater age than 12 hours probably should not be tested. It is also unknown whether this method would distinguish smaller differences in the rate of growth in series whose members differed by small increments. With the wide zones tested, the method gave satisfactory results.

II. Comparison of "general growth condition" of different cultures of the same streptococcus.

This set of conditions does not require as strict definition as the former. "Lag" and similar phenomena involved in bacterial growth and acid production do not limit the value of the method in this instance. In fact, they are of actual service in the application of the method for the comparison of the "general growth condition" of a series of cultures of the same fermenting agent.

The "general growth condition" is assumed to represent not only the number of viable cells but also their relative vitality and activity. The number of cells and also their condition, is dependent upon the environment and upon the period to which they have been exposed to this environment. Both of these relations are involved in the speed of multiplication and of acid production of inocula taken from a series of different environments.

Hence, under this set of conditions, the method can be applied in a comparison of the relative influence of different environments for a given period of time. It may also be used in the comparison of the influence of exposure to a given environment for different periods of time.

Both of these relations are evident in the experiment in which the second set of conditions has been maintained, in a study of the relative influence of different temperatures upon the growth and physiological activity of different types of streptococci. This method should furnish a ready means of comparing the "general growth condition" or vitality of different cultures of the same strain. Both rate of acid production and multiplication are dependent on the condition of the inoculum (age, size, and resistance to previous environment), which is in fact the definition of the "general growth condition" or vitality.

It is probable that this method furnishes a more direct evaluation of differences in bacterial vitality than can be furnished by the plate method. The later method merely presents an approximation of the relative number of viable cells present which are ultimately able to produce a colony of appreciable size, while the above described method presents an approximation of the relative condition of the cells.

e. Disadvantages of the method.

The method at best is crude. It can serve only in the comparison of the same strain, as differences exist in the fermenting capacity of different strains. The introduction of large inocula into the glucose Andrade broth must be avoided, in cases where significant changes in the reaction of the broth would be effected in that way. The choice of Andrade indicator is open to serious questions. The change in color of this indicator is not instantaneous by any means. Exact determinations of H-ion concentration cannot be made. (It is probable that more accurate data could be obtained by the use of brom cresol purple and a definitely standardized colorimetric comparison.)

f. Advantages of the method.

The chief advantage of the method is its convenience. Less time, labor and materials are required than for carefully controlled plate counts.

Disadvantages following the choice of Andrade indicator have been mentioned. However, it has the following advantages. The presence of this indicator in the glucose broth apparently exerts

no harmful influence upon the growth of streptococci, as this indicator is commonly used in tests of carbohydrate fermentation. The fact that Andrade indicator changes in color at H-ion concentrations not far removed from the neutral point is also a point in its favor. (Tennel and Fisher reported definite magenta color is exhibited by Andrade indicator at a ph of 6.6 to 6.8.)

There are two reasons for the use of an indicator whose color change is not far removed from the neutral point. (1) At high H-ion concentrations the products of growth are imposing their influence upon the curves of growth and of acid production. Hence, acid production is a function of the initial number of cells only during the early phase of the fermentation. (2) At later periods in the course of fermentation, the differences in concentration of acid produced by inocula of different sizes are constantly decreasing. The greatest differences are manifest in earlier periods of the fermentation. (This relation is evident in the results reported in the work of Foster (p. 182, fig. 3.), which appeared several months after the completion of this investigation. The relations involved here are also discussed in detail under "Theoretical Progress of Lactic Acid Fermentation" in Part I of this paper. Curves given there also furnish further support to the above statements.)

The method is not presented as a method, but merely as the means by which results reported in this study were obtained. In the following text, this method is termed the "Andrade Test".

INVESTIGATION.

Section A.

THE RELATIVE INFLUENCE OF ENVIRONMENTAL CONDITIONS UPON THE LIFE PROCESSES OF DIFFERENT TYPES OF STREPTOCOCCI, FOR THE PURPOSE OF STUDYING THEIR PEPTOLYTIC ACTIVITY.

I. Influence of H-ion Concentration Upon the Peptolytic Action and Other Life Processes of Different Types of Streptococci.

The influences of H-ion concentration upon the functioning of micro-organisms have proven fruitful subjects of study. These investigations have shown that the true acidity of the environment conditions, and, in many cases, determines, the extent and direction of the various processes involved in microbial metabolism.

In a study of the proteolytic activity of different types of streptococci, the influence of this factor assumes particular moment. The sphere of action of their proteolytic enzymes is limited to certain zones of H-ion concentration; again, even within the zone permitting their action, the degree of activity is conditioned by smaller increments of change in the true acidity of the system. The proteolytic enzymes can not function until they are first elaborated and their elaboration is dependent upon the successful and luxuriant growth of the cells. Consequently, the influence which H-ion concentration exerts upon all of the life processes of streptococci is reflected in a less direct but still pertinent manner, upon the proteolytic changes induced by their enzymes. All of these relations project themselves into many

processes in the different fields in which streptococci are important.

1. Influence of H-ion concentration upon amino nitrogen increases.

The influence of the H-ion concentration upon the increases in amino nitrogen effected by the different types of streptococci, was studied by comparing the changes brought about by the type strains in systems of different pH value. Comparatively wide zones were chosen as a means of determining variations in the general behavior of streptococci in different H-ion concentrations. Changes in the initial reaction of even highly buffered media occur in plain broth cultures of streptococci, which would render very difficult the comparison of the influence of initial differences of smaller increments of pH. (Itano, 1916).

Procedure: (Experiment 1.)

Preparation of medium: Infusion broth prepared as follows was used as the medium in this experiment:

For each desired liter of medium, 500 g. chopped lean meat was added to 1,000 cc. of distilled water. After the mixture had been infused 12 hours in the ice-box, it was strained thru cheese cloth. The infusion was heated one half hour at 100° C. and then filtered. The volume was corrected and the infusion autoclaved in flasks. One per cent of Difco peptone and 0.5 per cent NaCl was added to the meat infusion. After ingredients were in solution, the broth was autoclaved for 5 minutes at 15 lb. pfessure and then filtered. The filtered broth was then sterilized in 600 cc. quantities, at 116° C.

The broth was adjusted to the desired pH zones as follows: Sample flask of the unadjusted broth was titrated colorimetrically, to the desired pH values by use of the standard solutions and indicators described by Clark and Lubs (1917) for the respective pH ranges.

The calculated amount of 1.0 n HCl or 1.0 n NaOH was added to the respective flasks of broth. Amounts added are given in Table I. Flasks were then shaken and incubated at 37o C. for

several hours.

The broth was then distributed into flasks in 100 cc portions, with aseptic precautions. These test flasks were incubated at 37° C. to insure sterility and to stabilize the medium. After 48 hours incubation, the H-ion concentration of the medium was determined electrometrically thru the kindness of Dr. A. Itano.

Table I.

Preparation of Broth of a Series of pH Zones.

Infusion broth, unadjusted, was autoclaved. Following amounts of alkali and acid were added with aseptic precautions. pH determined after 2 days incubation at 37° C. pH values represent the H-ion concentration at time of inoculation.

Desired pH Zone	Amount added per 100 cc. of unadjusted broth.		Actual pH.
	1.0 n HCl	1.0 n NaOH	
4.5	1.40	----	4.45
5.5	0.50	----	5.40
6.5	----	1.05	6.70
7.5	----	1.70	7.35
8.5	----	3.12	8.63

(Experiment 1.)

One flask of medium at each pH value was inoculated with 0.1 cc. of an 18-hour broth culture of the representative strain. The cultures were then incubated at 37° C. Samples were removed at the intervals stated and determinations made of the amino nitrogen.

The results are given in Table II.

The significance of these results will be discussed in the general review of the experiments upon the influence of H-ion concentration upon the life processes of different types of streptococci.

Table II.

Influence of H-Ion Concentration upon Amino Nitrogen Increases.

Broth of different pH value received inocula of 0.1 cc. of an 18-hour broth culture of each type strain. Results of NH_2-N determinations are expressed below in mg./100 cc.

	pH Zone.*	Total NH_2-N.			Increase in NH_2-N.
		24 hr.	168 hr.	Control.	
Lactic	5.5	54.3	55.7	53.5	2.2
	6.5	54.0	56.5	52.2	4.3
	7.5	52.7	55.6	51.9	3.7
	8.5	51.1	51.2	51.2	---
Human	5.5	53.3	53.6	53.5	---
	6.5	53.0	59.5	52.2	7.3
	7.5	53.6	60.8	51.9	8.9
	8.5	52.0	51.8	51.2	0.6
Bovine	5.5	53.0	55.5	53.5	2.0
	6.5	53.9	55.5	52.2	3.3
	7.5	53.3	55.9	51.9	4.0
	8.5	51.3	51.5	51.2	0.3
Cheese	5.5	54.8	56.8	53.5	3.3
	6.5	57.0	61.7	52.2	9.5
	7.5	56.5	60.2	51.9	8.3
	8.5	51.1	52.1	51.2	0.9

*Actual initial pH values of the series are given in Table I.

2. Influence of H-ion concentration upon the growth and viability of different types of streptococci.

In such tests as those just reported, the influence of different initial pH zones upon "proteolytic" action is to a large extent a reflection of the effect of initial reaction upon growth. The following experiments were conducted to show in a more specific manner, the influence of different initial H-ion concentrations upon the growth and vitality of the different streptococci.

Procedure:

(Experiment 2). Acid Tolerance.

In the preceding experiment, the cultures at pH 4.5 had shown no growth either by turbidity or by increases in NH_2-N. In this experiment the test of the influence of this reaction zone was limited to test of the vitality of the different types after varying periods of exposure to this zone of H-ion concentration. Plates were poured at stated intervals, to give some idea of the approximate rate of death of the different types. No attempt was made to follow the course of the killing reaction. This series received inocula of 1.0 cc. of 18-hour broth cultures.

Plate counts were made of the cultures from which these inocula were taken. The data obtained indicated that the following number of cells represents the initial concentration of bacteria per cc. of the test broth. *

Lactic	400,000
Human	60,000
Bovine	120,000
Cheese	600,000

*The variation in the number of cells represented in the above figures is more apparent than real. It is probable that the actual initial concentration of the cells of the different streptococci is much more uniform than would appear from the above figures. These differences in numbers as determined by plate counts are due to recognized and inherent errors in counts made by the plate method. The divergence in numbers is exactly what would be expected from the difference in length of chains represented by the different type strains. The much lower values always obtained in plate counts of long-chained streptococci (such as the above human and bovine strains) is a common laboratory observation. As the number of cells is compared only with numbers of cells of the same strain, this apparent discrepancy in the initial concentration of the different streptococci does not vitiate to any extent the value of the results given in Tables IV and V.

As a more ready means of comparison of the resistance of the different types to high H-ion concentrations, the counts made in the tests have all been reduced to a common basis of an equal initial concentration of 100,000 cells per cc. The results presented in Table IV are expressed upon this basis.

(Experiment 3.) Rate of growth and longevity.

In the other pH zones, plates were poured at the intervals shown in Table V, in an attempt to present comparative figures representing the relative rate of growth of the different types when introduced into systems of different pH values. The tests made at the later periods furnish some idea of the relative longevity of these types. Cultures in this series were inoculated with 0.1 cc. of 18-hour broth cultures.

Results are given in Table V.

Plate counts were made of the cultures from which inocula were taken, at the time of inoculation of this series. Numbers so obtained were divided by the number of cc. of the test culture. The result is expressed in the table as "Probable initial number of cells present".*

Media used:

Media used for the tests: Broth of a series of pH values were prepared as described in the preceding experiment, except that the colorimetric method was used in determining the H-ion concentration. This broth was distributed into flasks in 50 cc. portions.

Media used in determining the number of cells: 1.0 per cent glucose infusion agar, pH 7.4, was used in making the counts. (This medium supported the growth of all strains.) In the pH 4.5 series, tests for vitality were also made by introducing 0.1 and 1.0 cc. of the inoculated broth into 10 and 50 cc. of glucose infusion broth, pH 7.6, containing 0.2 per cent sodium phosphate.

Table IV.

Relative Acid Tolerance of Different Types of Streptococci.

Comparison of the viability of different streptococci in pH 4.5 broth. Number of cells viable per 100,000 of initial concentration, after the stated intervals of exposure to pH 4.5 at 37° C.

Exposure. (hrs.)	Human.	Bovine.	Lactic.	Cheese.
5	100	9,000	48,000	90,000
10-12	---	1,200	10,000	120,000
24	---	-----	800	66,000
72	---	-----	------	80,000
120	---	-----	------	30,000

Final H-ion concentration in glucose broth cultures of the same strains.

pH	5.0	4.4	4.2	4.1

*The statements made in footnote on preceding page will explain the apparent divergences in number of cells used as inocula.

a. Relative acid tolerance of the different types:

The results given in Table IV show the relative resistance to high H-ion concentrations exhibited by the different types of streptococci. The order of resistance of the different types to high acidities is as follows: cheese, lactic, bovine and human.* The actual rate of death, of course would be less at lower temperatures than at 37° C., but it is doubtful if this would displace the above relations between the different types.

As the pH value of the medium used in this experiment closely approaches the acidity of sour milk, the above order of acid resistance of the different streptococci possesses obvious significance. In such a medium, one would expect the human streptococcus to die rapidly. The lactic and cheese strains would persist longer than the bovine or mastitis types. The cheese strain is much more resistant than the lactics and would not decrease in numbers so rapidly in sour milk, acid cheeses and similar acid systems.

*While it is true that "plate counts" seemed to show a great divergence in initial concentration of cells, it must be remembered that these large differences are due to the plate method itself, which enumerates clumps and groups as single organisms. It happens that these differences are also in the same order as the relative acid tolerance. One must admit the possibility of influence of the size of the inoculum and the initial concentration of the cells upon the rate of death of cells, but it is obvious that slight differences in initial concentration can not produce such wide divergences in time required for the end-point of the killing reaction, as are represented in Table IV.

These general relations to high acidity are more or less in order with the final H-ion concentrations reached in glucose broth cultures of the different types. From the final pH attained by the human strain, its rapid rate of death in broth of higher H-ion concentrations is to be expected. However, the specific effect of the H-ion is not so readily interpreted as to permit the assumption of parallel relations between the final "fermentation limit", and the tolerance of the organism to high acidities when introduced as inocula from cultures which have been growing in the optimum pH zone. In experiments with the pneumococcus, Avery and Cullen (1919 b) have demonstrated this in a conclusive manner. (These relations are discussed in detail in Part I of this paper, under "Influence of H-ion Concentration upon the Lactic Acid Bacteria", and "End Point of Lactic Acid Fermentation".) This is evident in a comparison of the final H-ion concentrations of the bovine, cheese, and lactic strains.

That no parallel relation exists between the final "fermentation limit" in glucose broth and the absolute tolerance of the organism to high acidities, is especially obvious in the case of the cheese and lactic strains. The cheese strain has a "fermentation limit" of pH 4.1 as compared to pH 4.2 exhibited by the lactic strain. In spite of the slight difference in this character, there is a striking difference in the actual resistance of these two strains in broth of pH 4.5 value. (It is shown in Table IV that the cheese strain was not greatly reduced in numbers in the pH 4.5 broth at the end of the time required for complete disinfection of the lactic, under the conditions of this experiment.)

The general relations in the acid tolerance of these types of streptococci are all that may be interpreted from the above experiment- and these, to a certain extent, only for the conditions obtaining in this particular experiment. Similar general physiological relations between the H-ion and streptococci, which are suggested by the above results, are also encountered in the results obtained in the other pH zones reported in Table V.

Table V.

Influence of H-ion Concentration upon the Relative Rate of Growth and Longevity of Different Types of Streptococci.

Broth of a series of different pH values received equal inocula of the different strains. Figures in table below represent the number of cells present after stated periods of incubation at 37° C.

Time (hrs.)	pH 5.5	p H 6.5	pH 7.5	pH 8.5
actic	(Probable initial number of cells present;--250,000.)			
5	900,000	4,000,000	3,200,000	360,000
12	20,000,000	80,000,000	55,000,000	900,000
24	60,000,000	95,000,000	60,000,000	200,000
72	3,400,000	7,400,000	6,000,000	350,000
120	18,000	15,000	12,000	11,000
168	4,000	8,400	9,000	2,000
nman	(Probable initial number of cells present:--90,000.)*			
5	50,000	750,000	1,200,000	190,000
10	500	1,400,000	3,200,000	250,000
15	20	1,900,000	1,500,000	140,000
24	------	1,700,000	2,000,000	100,000
48	------	800,000	1,400,000	12,500
72	------	200,000	600,000	-------#
120	------	1,600	2,000	700
168	------	800	750	320
evine	(Probable initial number of cells present:--100,000.)*			
5	200,000	900,000	1,500,000	150,000
10	500,000	2,000,000	2,600,000	180,000
24	5,200,000	5,400,000	9,500,000	100,000
48	3,000,000	2,300,000	4,000,000	160,000
120	---------#	18,000	12,000	1,000
168	---------#	10,000	16,000	300
neese	(Probable initial number of cells present:--600,000.)*			
5	2,800,000	7,000,000	5,600,000	1,200,000
10	36,500,000	280,000,000	200,000,000	20,000,000
24	400,000,000	560,000,000	500,000,000	15,000,000
72	600,000,000	280,000,000	350,000,000	9,500,000
120	38,000,000	150,000,000	110,000,000	6,300,000
192	1,800,000	95,000,000	73,000,000	3,000,000

\# Plates contaminated, but streptococcus colonies present; glucose broth tests, positive.

* Recall footnotes, pp.

b. Influence of H-ion concentration upon the rate of growth and longevity of different types of streptococci.

The results given in Table V show the following general relations.

Zone showing maximum growth: The lactic strain exhibits its maximum rate of growth in media of a reaction representing a zone between pH 6.0 and pH 7.0. The maximum growth of a lactic strain in a similar zone has been reported by Itano from observations of turbidity, and more recently by Svanberg. The cheese strain also exhibits its maximum growth in this zone. The fact that this zone of most rapid growth for the lactic streptococcus approaches the pH value of milk has been pointed out by Itano. The human and bovine strains grow most rapidly in the zone between pH 7.0 and 8.0.

Comparative growth in limiting zones: In the zone representing pH values 8.0 to 9.0, the cheese strain is the only type which increased in numbers to a significant degree. Since this strain also exhibited the greatest acid tolerance in the experiment reported in Table IV, it is evident that this type possesses considerable resistance over a wide range of pH. Again, in the zone representing pH values 5.0 to 6.0, the cheese strain grows more rapidly than any of the other type strains.

The general significance of these relations and their comparison with the amino nitrogen increases in a similar series of pH values, will be given in the general discussion of the influence of H-ion concentration upon the different streptococci.

We wish to take this occasion, however, to point out the behavior of the human strain in broth of approximately pH 5.5. This streptococcus reaches a H-ion concentration of pH 5.0 as its

"fermentation limit". However, when inocula taken from cultures growing near the neutral point are introduced into plain broth of lower true acidity, they not only are not able to initiate growth, but rapidly decrease in numbers. The same relation is also shown by the bovine and lactic strains in the pH 4.5 test reported in Table IV. Hence, acidities less than their final "fermentation limit" appear to be not tolerable for the initiation of growth and, in fact, to lead to the death of the cells.

No extended discussion of these relations is warranted from our results. The distinction between the actual "fermentation limit" and the H-ion concentrations serving for the initiation of growth of the pneumococcus was shown by Avery and Cullen. The same distinction apparently exists in the case of streptococci. (The relation and distinction between the "fermentation limit" and the "limiting H-ion concentration for initiation of growth" have been discussed in detail in Part I of this paper.)

Relative longevity in different H-ion concentrations within the range of growth: The relation of H-ion concentration to the longevity of these strains cannot be interpreted directly from the above data. The rate of death in old cultures of any H-ion concentration probably follows a more or less orderly course. Consequently, the number of viable cells present at the time of any of the later analyses is dependent upon the number present in the period of maximum growth, as well as upon the H-ion concentration of the system. Moreover, in the above pH zones, in which growth has taken place at a variable and unequal rate, the concentration of metabolic products (upon which the rate of death is also dependent) probably is significantly different. Hence, there are too many factors involved in the determination of the numbers viable after long exposure to the different pH zones of growth, to permit the assignment of any specific effect to the hydrogen ion.

5. Summary and discussion of the influence of H-ion concentration upon the life processes of the different type strains.

a. Influence upon amino nitrogen increases.

The lactic strain splits peptone most actively in broth between pH 6.0 and 7.0. The cheese strain seems to require the same optimum zone for its most successful peptone attack, but its activity is inhibited to a much less extent than in the case of the lactic strain. The bovine and human strains prefer a pH zone 7.0 to 8.0 for peptolytic activity.

The increases in amino nitrogen evidenced in systems of different pH value are dependent upon several factors and the concentration of H-ion may exert a quantitatively different influence upon each of these factors. The influence of pH upon the growth of cells is one determinant of the concentration of the enzyme, and is thus involved in the determination of the rate of amino production. However, the final increase in amino nitrogen in systems of different pH value is more often a reflection of a number of simultaneous influences of the H-ion upon a number of other factors.

Chief among the factors so involved are the effect of the H-ion upon the activity of the enzyme or enzymes themselves, and the influence of the H-ion concentration upon the liberation of enzymes. Again, there is always a possibility of more than one enzyme being involved in the breaking down of protein derivatives, and the specific effect of the H-ion upon the members of such a possible enzyme complex may be decidedly different. (Dernby). It has also been shown that H-ion concentration exerts a significant influence upon the disintegration of bacterial cells and consequently, upon the liberation of endoenzymes. With these relations in mind, the assignment of a specific role to the H-ion in the determination of the amino nitrogen increases can not be approached without corresponding tests with enzym solutions obtained from the strains involved.

Practical significance: The practical significance of the above results probably consists in the following relations. In systems of pH value approximately 5.5, the constituents of "peptone" are broken down more actively by the hemolytic cheese strain than by any of the other type strains. This suggests that members of this group may play a significant part in changes in the distribution of nitrogen in acid cheeses and in other dairy products, the H-ion concentration of which would inhibit the activity of true lactics to a greater extent.

b. Influence upon growth.

Optimum pH zones for growth: With the large pH increments tested in the above experiments, there was apparent agreement between the optimum zones for the splitting of peptone and for growth of cells.

Limiting initial H-ion concentrations: Each of the type strains proved unable to initiate growth in systems of lower true acidity than the final acidity reached in the glucose broth cultures of the same strain. Comparatively rapid death of the streptococci occured in the zone which apparently lies between the usual "fermentation limit" and the "limiting H-ion concentration for initiation of growth".

Acid Tolerance: When introduced into broth pH 4.5 at 37° C., the type strains exhibit the following order of acid tolerance: cheese, lactic, bovine, and human. Altho this is the same order as in the case of their final H-ion concentration in glucose broth, it is evident that the so-called acid tolerance shows a

much greater divergence than would be expected from a comparison of the final pH reached in cultures of the same strain. Such a relation indicates that the final H-ion concentration or "fermentation limits" are not due merely to an intrinsic resistance to the bactericidal action of the H-ion.

Practical significance: The practical significance of these results lies in their projections to the illumination of the rôle of H-ion concentration in the determination of the moment of these different types of streptococci in the microbial balance in milk and milk products. These relations are important. An intelligent interpretation of the significance of the presence of different types of hemolytic streptococci in cheese and other dairy products, requires some knowledge of the resistance of these microorganisms and of their ability to grow in the acidities presented in acid milk products

Given a sample of fresh milk in which all four of these types of streptococci were present, the more rapid rate of growth of the lactic and cheese types in systems of H-ion concentrations between pH 6.0 and 7.0, would explain the fact that they rapidly outgrow the human and bovine streptococci.* The greater sensitivity of the human and mastitis strains to high acidities would tend to cause their disappearance in sour milk, butter and certain cheeses.

*No intimation is made that the initial limiting pH zones are the same in milk as in plain broth. Avery and Cullen have shown a decided difference in the H-ion concentrations permitting the initiation of growth of the pneumococcus in sugar-free and in sugar broths. However, it is doubtful if the relative rate of growth of the different types in different pH zones would be greatly disturbed by a change of medium. Nevertheless, the possibly selective influence of unknown factors in milk cannot be ignored.

The above suggestions are in keeping with common observations, such as those recently reported by Jones on the decrease in relative numbers of hemolytic udder streptococci in milk upon incubation. They are also in accord with the usual assumption that the hemolytic mastitis or udder streptococci are comparatively or relatively frequent in certified milk or in fresh market milk, but are outgrown by the common lactic in market milk at later periods of its handling and in fermented milk products.

The resistance of the cheese strain to high acidities would explain its appearance in significant numbers in milk products of high acidity, even though it were originally present in relatively small numbers.

II. Influence of the Stage of Growth of the Culture upon the Increases in Amino and Ammonia Nitrogen.

1. Experimental

The results in the preceding experiment suggested that a significant part of the increase in amino nitrogen occurred after the cessation of growth of the cultures. The present experiment attempts to compare the increases in amino nitrogen and ammonia nitrogen at different periods of the life history of cultures of streptococci.

Procedure: (Experiment 4.)

Infusion broth, containing 0.2 per cent sodium phosphate was prepared in two lots of pH values 6.6 and 7.4. 100 cc. of the pH 6.6 broth was inoculated with the lactic strain; the same quantity of the pH 7.4 broth, with the human strain. These were incubated at 37° C. Samples were removed at the end of 1, 3, 5, and 10 days. Plate counts and NH_2-N determinations were made at each of these intervals. NH_3-N determinations were made from the 3 and 10 day samples.

Results are given in Table VI.

Table VI. a.

Influence of Stage of Growth upon Increases in Amino and in Ammonia Nitrogen.

	Time	NH_2-N		NH_3- N		Growth Stage
	days	mg.-in 100 cc.		mg.-in 100 cc.		Thousand cells in 1 cc. of culture.
Lactic		Total	Increase	Total	Increase	
	0	55.1		9.8		
	1	57.0	1.9			140,000.
	3	58.5	3.4	16.5	6.7	80,000.
	5	58.8	3.7			80.
	10	59.5	4.4	16.7	6.9	4.
Human						
	0	53.2		9.8		
	1	56.2	3.0			6,000.00
	3	58.6	5.4	16.3	6.5	2,000.00
	5	60.4	7.2			8.00
	10	62.0	8.8	17.1	7.3	.85

Table VI.b.

Comparison of Increases in Amino and Ammonia Nitrogen During Early and Later Periods of History of the Culture.

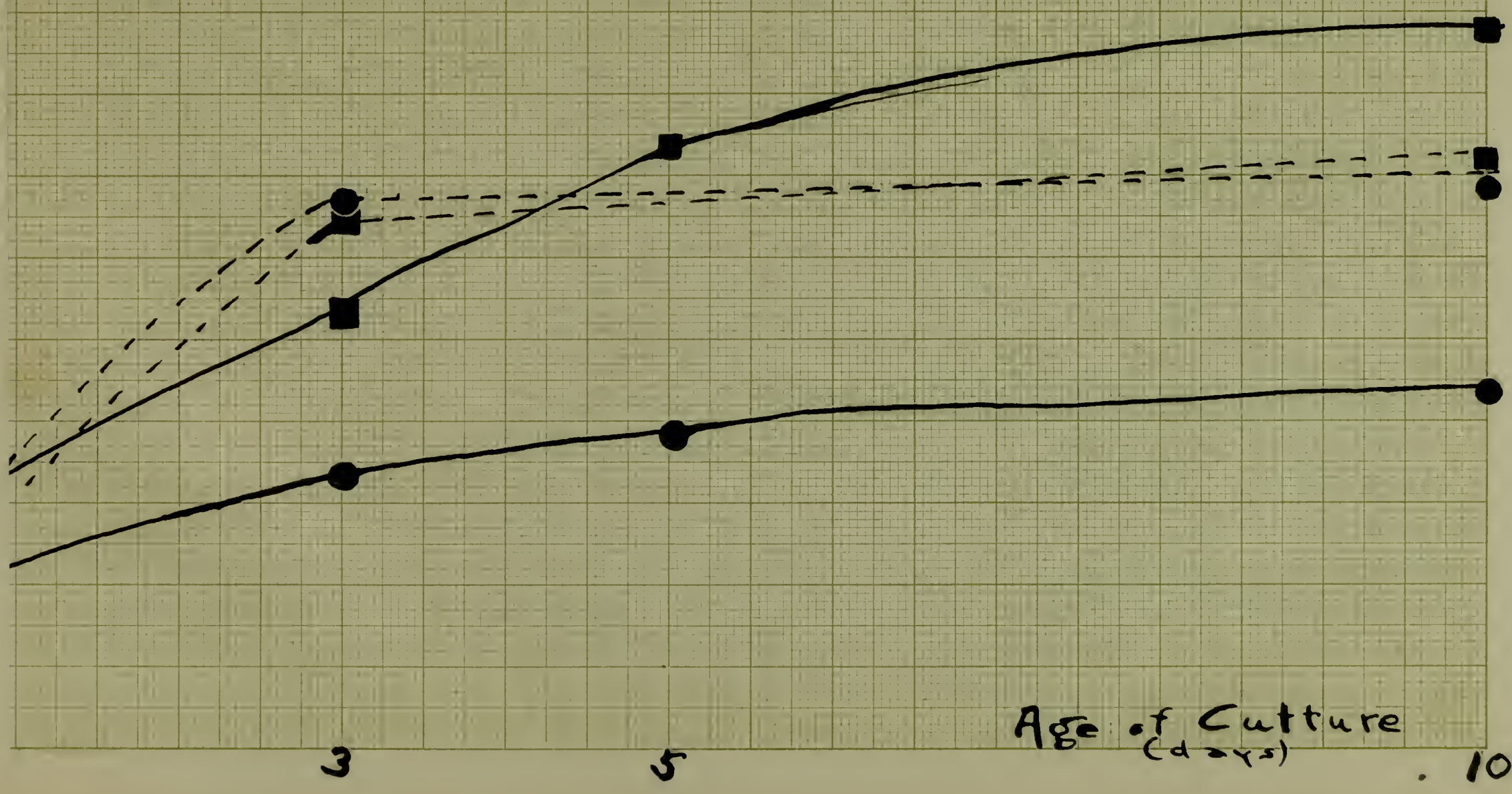
	Amino Nitrogen Increases		Ammonia Nitrogen Increases	
	First 3 days	3rd to 10th day	First 3 days	3rd to 10th day
Lactic	3.4	1.0	6.7	0.2
Human	5.4	3.4	6.5	0.8

Relation of Stage of Growth of Cultures to Increases in NH_2-N and NH_3-N.

Figure 1.

(values plotted below are taken from Table VI.)

— NH_2-N ■ Human Strain
-- NH_3-N ● Lactic Strain.



It is evident in Table VIa that the cultures continue the formation of amino compounds considerably beyond the period of growth. In Table VIb it is shown that the increases in the amino nitrogen after the third day in the history of the culture represent a significant part of the total increase. During the same period, a much smaller proportion of the total increase in ammonia is formed than in the case of the amino nitrogen. This is also shown in Figure 1. A general discussion of the possible meaning of these relations will be given below.

2. General discussion of the relation of growth stage of cultures to increases in amino and ammonia nitrogen.

a. Nature of nitrogen distribution in peptone broths.

As is well known, commercial peptone contains a greater variety of peptone deriviatives than might be inferred from the use of the term "peptone". A certain amount of the total nitrogen is found in the form of compounds which are precipitated by the usual protein precipitants. This precipitated portion would be termed "protein nitrogen". In the same sample of peptone, the proportion of the total nitrogen to be termed "protein nitrogen" will vary with the precipitant used.(A. Hiller)*. When precipitation is effected by colloidal iron, the "protein nitrogen" would consist of proteins and certain protein deriviatives of only slightly less complexity (Van Slyke, Vinograd-Villchur, and Lossee). The filtrate would contain the nitrogenous constituents of lower complexity, and would be termed"non-protein nitrogen". This fraction would then be separated into "peptide", amino, and ammonia nitrogen. The

*Unpublished manuscript by Van Slyke and Hiller.

peptide nitrogen would consist of that portion of the non-protein nitrogen which can be hydrolyzed to amino compounds. The amino nitrogen and ammonia nitrogen would consist of the portions of the non-protein fraction which can be determined as amino and ammonia compounds respectively.

In beef infusion peptone broth)culture medium used in this study) a further contribution to the total nitrogen is made by the infusion itself. A large part of its total nitrogen is in the form of amino compounds. (Infusion broth always contains a larger amount of amino nitrogen than does extract broth.)

The actual distribution of the nitrogen contained in peptone will, of course, vary with the degtee of hydrolysis to which the product has been subjected in its manufacture. (It is a common observation that the usual American products contain a larger proportion of simple constituents than does the Witte product. Difco peptone used in this study, is perhaps one of the furthest hydrolyzed of the American products.)

b. Meaning of amino nitrogen and ammonia nitrogen increases in bacterial cultures.

From the above paragraphs it is seen that preformed amino and ammonia nitrogen exist in the culture medium before its inoculation. Both of these classes of nitrogenous substances are utilized as food by microörganisms. In the early period of the culture, the microörganisms are largely dependent upon the preformed simple nitrogenous constituents presented in the medium for the nitrogen portion of their food, whether used for energy or for growth. In all probability, the utilization of a certain amount of these substances precedes the actual formation of either. It is evident that both amino and ammonia compounds are utilized as well as formed by microörganisms, and that the utilization and formation of these compounds may preceed simultaneously and at constantly changing velocities.

With these facts in mind, it is obvious that determinations of either of these compounds, if made in the early history of cultures in media containing preformed amino and ammonia nitrogen, present more or less incidental values. Increases observed in their total concentrations merely represent the formation of a greater amount than has been consumed. The complexity of these relations is increased by the fact that the portion of amino nitrogen consumed may be taken either from that preformed in the system or from that which has been formed by the organism itself. From these relations, it follows that determinations made in the early history of cultures which do not actively and at an early stage attack peptone with the formation of amino and ammonia compounds, will show an actual decrease in the total concentration of these substances. Such findings are not infrequent. (No tests in the early periods of cultures have been made in this study. However, in Table XV I are presented results obtained with one strain which showed a slight decrease in amino nitrogen even after 10 days incubation; this strain did not show increases in amino nitrogen until cultures were several weeks old.)

In the above data, Table VIa, the increases in amino nitrogen in the 24-hour tests must be considered as representing the difference between the amount of amino nitrogen which has been formed and that which has been consumed in that period. The same probably applies to the 72-hour values but to a less extent as a decrease in the number of active cells is evident. The increases observed in the 5- and 15-day tests would seem to have occurred during a period in which little or no utilization of food would take place. The fact that the ammonia increases seem to be limited more closely to the earlier history of the culture suggests that ammonia production is more strictly

associated with the actual growth of streptococci.

The drawing of definite conclusions on the significance of these apparent relations is impossible in the face of the following possible complex sources, and methods of formation of amino and ammonia compounds in peptone broth cultures.

c. Possible sources and methods of formation of amino and ammonia compounds in peptone broth cultures of streptococci.

From the nature of nitrogen distribution in peptone broth, it follows that amino compounds may arise from the hydrolysis of a number of compounds of different nature. Both the peptide and protein fractions are capable of yielding amino acids,--moreover, amino nitrogen may be split off at the successive stages of their cleavage. It has been shown above that, during certain stages of the cultures, the utilization of amino compounds proceeds simultaneously with their formation. Hence, the utilization of amino compound may involve attacks upon the preformed amino nitrogen. or upon that formed by the organism or by its enzymes from any of the substrates which yield amino acids. Thus, the increase in amino nitrogen observed at any time represents the difference between the amount of amino compounds formed from any of the potential substrates and the amount of amino nitrogen consumed by the microorganism during the same period.

The production of amino acids may be assumed to be due to the hydrolytic action of the enzymes of the streptococci. However, many questions which are involved in the relation of the formation of amino nitrogen to the actual growth and metabolism of streptococci are unanswered today. The same is true of a satisfactory interpretation of the complete mechanism of its microbial formation. It does not seem that the formation of amino

acids can be considered merely as a waste product of microbial growth nor that their production is limited to periods in which growth is taking place. Neither does it seem that their production can be separated from the processes involved in the growth and life of the cells. However, it is probable that the total increases in amino nitrogen may be considered as including at least two general periods of formation, as based upon their relation to the growth of the culture. (It is understood, that such a division can be only approximate.)

A certain part of the total increase probably represents an unused residue of the total amount of amino compounds formed during the active growth of the culture. It is only reasonable to suppose that the living cell manifests at least a quantitative selection of amino acids from the collection presented to them (by the sum of the hydrolytic products of their enzymes and by those preformed in the system). The increase in amino nitrogen during the growth of the culture represents this unused residue, a part of which possibly can be considered as the portion less desirable to the organism, and a part merely as an excess production. These conditions would, of course, prevail only during the period of growth and activity of the cells themselves.

However, the above experiment shows that the production of amino compounds is by no means limited to the period of growth as the concentration of amino nitrogen increases during the period in which the cells are dying. The formation of amino compounds in this period may be regarded as due to the more or less incidental action of their "proteolytic" enzymes, whose activity often persists after the death and disintegration of the cells which elaborated

them. Hence, a certain part of the total production of amino acids is apparently due to the action of liberated enzymes,--more independent of the needs of the cell than in the former case. In this period probably all of the amino acids formed appear in the total increase of amino nitrogen in the system.

The production of ammonia by bacteria has been ascribed to the intra-cellular deaminization of nitrogenous food by the microorganisms (Kendall and Walker). This relation would indicate that ammonia formation is more closely associated with the growth and actual life of the cell than is the production of amino compounds. The results obtained in the above experiment are in keeping with this conception. Altho it is probable that the greater part of the total increase in ammonia is intimately associated with the metabolism of the cell, it is unfair to ignore the appearance of a small amount of ammonia as a possible cleavage product of the activity of hydrolytic enzymes.

III. Influence of Temperature Upon the Peptolytic Action and Upon Other Life Processes of Different Types of Streptococci.

The influence of temperature as a factor in the environment of lactic acid bacteria has been reviewed in detail in Part I of this paper. There it was shown that temperature may be a conditioning factor of the rate, the extent, and at times, the direction of microbial processes. ("Influence of Environment upon the Lactic Acid Bacteria"; "Theoretical Progress of Lactic Acid Fermentation".)

With these relations in mind, a study was made of the relative influence of different temperatures upon the life processes of the different types of streptococci. The pertinence of such a study

is directly evident from the significance of temperature in the systems in which these different streptococci are found. The direct significance of these temperatures upon the different types of streptococci will be discussed in the interpretation of the results of the present study.

1. Influence of different temperatures upon amino nitrogen increases.

The influence of different temperatures upon the amino nitrogen increases effected by the different types of streptococci was studied by comparing the changes brought about by the type strains in the same system under different temperature conditions. The temperatures of 15°, 23°, 31°, and 41° C., were chosen as test temperatures.

As a rough index of the influence of temperature upon the rate of peptone splitting, determinations were made of the amino nitrogen increases brought about by equal inocula after 24 hours incubation at different temperatures. As a means of comparing the influence of temperature upon the final increase in peptolytic products, determinations were made of the final increases in amino nitrogen after longer periods of incubation.

Procedure: (Experiment 5)

Infusion broth, pH 7.2, containing 0.2% sodium phosphate, was sterilized in 100 cc. portions. Series of flasks of medium were placed in constant temperature rooms at 15° and 23° C.; other series were placed in incubators at 32° and 41° C., respectively. The media were held over night at these temperatures before inoculations were made.

Flasks of the different series received equal inocula of the respective strains, and were then incubated at the designated temperatures. The 23° series varied in temperature from 22-23° C.; the 15° series, from 14-15° C. NH_2-N determinations were made from 1, 5, and 15 day samples. Results are given in Table VII.

Table V II.

Amino Nitrogen Increases at Different Temperatures.

Medium: Infusion broth, pH 7.2, containing 0.2 per cent sodium phosphate. Flasks of each temperature series received equal inocula of the respective strains. Results expressed below as mg. NH_2-N in 100 cc.

	1 day	5 days	15 days	Increase in NH_2-N.
Human				
15°	----#	55.4	55.5	---
23°	56.4	63.9	65.0	9.5
31°	57.9	64.1	65.4	9.9
41°	55.9	58.2	58.5	3.0
Bovine				
15°	----#	55.5	55.5	---
23°	57.0	57.5	58.7	3.2
31°	58.9	59.1	59.4	3.9
41°	58.8	60.0	60.1	4.7
Cheese				
15°	55.0	61.4	62.0	6.5
23°	60.0	63.0	64.3	8.8
31°	63.7	64.0	64.8	9.3
41°	63.9	64.8	65.3	9.8
Lactic				
15°	54.7	58.9	60.2	4.7
23°	57.8	59.1	60.6	5.1
31°	58.6	59.5	60.3	4.8
41°	57.5	58.6	58.8	3.3

Not determined.

The results given in Table VII show the following general relations.

Peptone splitting by the lactic strain proceeded most rapidly in the temperature zone approximating 30° C. Although the greatest rate is exhibited at this temperature, the final increase in amino nitrogen is greatest at a somewhat lower temperature. The human and bovine strains seem to split peptone most rapidly at temperatures between 30 and 40° C. The cheese strain, however, shows a greater rate of increase at a temperature of 41° than at 31° C.

The final increase in amino nitrogen is greatest in the 31° test in the case of the human strain. Significant increases occurred in the greatest range of temperatures in the case of the cheese strain, although the lactic strain is relatively more active at the lower temperatures. At a temperature of 41° C., the processes involved in the production of amino compounds are inhibited to a marked degree in the case of the human and lactic strains. This inhibition is exhibited both in the rate of peptolysis and in the final increase in peptolytic products. The bovine and cheese strains seem to exhibit their greatest increases in the 41° test. (This phenomenon is explained in the next experiment.)

The significance of these relations will be discussed in the general review of our study of the influence of temperature upon the life processes of different types of streptococci.

2. Comparison of amino nitrogen increases at 37° and 41° C.

The bovine and cheese strains had exhibited greater amino nitrogen increases at 41° than at 31° C., in the above experiment. While it did not seem probable that these strains would be more

active at temperatures slightly above 37° C. than at the usual incubation temperature, the possibility of a higher optimum temperature could not be ignored. Such a temperature relation would be peculiarly pertinent in the case of the bovine or mastitis strain as the usual body temperature of cows is higher than that of humans. To investigate such a possible relation, the following experiment was conducted:

Procedure: (Experiment 6)

Two series of flasks of 30 cc. of infusion broth (pH 7.2 and containing 0.2 per cent sodium phosphate), received inocula of 0.1 cc. of the human, bovine, and cheese strains. The series were incubated at the temperatures of 37° and 41° C., respectively. NH_2-N determinations were made at the end of 7 days incubation.

Results are given below in Table VIII.

Table VIII.

Comparison of Amino Nitrogen Increases at 37° and 41° C.

Medium: Infusion broth, pH 7.2, containing 0.2 per cent sodium phosphate.
Cultures 7 days old at time of analysis.

	Total NH_2-N. mg. in 100 cc.		Increase in NH_2-N. mg. in 100 cc.	
	37°	41°	37°	41°
Human	64.5	61.0	9.2	5.7
Bovine	59.0	58.6	3.7	3.3
Cheese	64.8	64.5	9.5	9.2
Control	55.3	55.3	---	---

The answer to the primary question involved in this experiment is evident in the results given in Table VIII. The total increases in amino nitrogen are less at 41° than at 37° C. in the case of all of the type strains. Hence, this temperature is above the optimum for the cheese and bovine strains. As in the preceding experiment the higher temperature does not inhibit their peptone attack to so marked a degree as in the case of the human strain.

It will be observed in Table VII that the bovine strain exhibited a greater amino nitrogen increase at 41° than at 31° C., while in Table VIII the increase is greater at 37° than at 41° C. The explanation of these relations is obvious. The temperature of 37° is apparently more near the optimum than is 41° C. This higher temperature depresses the peptolytic action to some extent, altho' even at this temperature, the bovine streptococcus is more active than at 31° C., a temperature considerably below its optimum.

It will also be observed that in this experiment the 41° test of the human strain is depressed to a greater extent than in the 41° test reported in Table VII. This depression happens to displace the order of increase among the different types, however, the same general temperature influence is still evident (the bovine and cheese strains are inhibited to a less extent at 41° than is the human strain). In the following experiment an investigation is made of one of the factors involved in the determination of the activity of the human strain at temperatures above 37° C. as a probable explanation of such observations.

3. Influence of size of inoculum upon amino nitrogen increases at optimum temperature, and at temperatures above the optimum.

In the several tests made of the amino nitrogen increases at 41° C., discrepancies frequently were evident. This was especially true in the case of the human strain (see above statements). In a search for the factors involved in the apparently irregular growth and peptone attack at this temperature, tests were made using different sizes of inocula. Tests made at 41° with inocula of different sizes frequently showed evident differences in the amount of growth, even when incubation was continued until cultures were sterile. Similar tests made at

37° C. showed no apparent difference. The following experiment is a report on one of these tests.

Procedure: (Experiment 7)

Infusion broth, pH 7.4, containing 0.2 per cent sodium phosphate, was used as the medium. Three flasks, each containing 60 cc. of broth, were placed in a water bath at 41° C. for 12 hours. They were then inoculated with the following amounts of a 12-hour culture of the human streptococcus: Flask 1, one loopful; flask 2, two two loopfuls; flask 3, 0.2 cc. This series was then immersed in a water bath held at 41° C. inside of an incubator, and incubated for nine days. The temperature did not vary more than 0.2° during the experiment. The tests were kept immersed so that the surface of the medium was at least an inch below the surface of the bath.

Two flasks of the same medium were inoculated with one loopful and 0.2 cc. of the culture used above, and incubated at 37° C. for the same period.

Observations of growth were made daily for four days, after which time all members of the 41° series were sterile. NH_2-N determinations were made at the end of nine days. Results are given in Table IX.

Table IX.

Influence of Size of Inoculum upon Amino Nitrogen Increases at Optimum Temperature and at Temperatures Above the Optimum, in Cultures of the Human Hemolytic Streptococcus.

Medium: Infusion broth, pH 7.4. Tests received inocula of sizes indicated below. The 41° series were sterile after 4 days. NH_2-N determinations were made at the end of 9 days.

	Amino Nitrogen mg. in 100 cc.	
Inocula	41°	37°
Control	53.0	53.0
1 loop	53.3	62.6
2 loops	53.6	
0.2 cc.	56.5	62.7

The results given in Table IX show that the amino nitrogen increases by this strain of streptococcus are severely conditioned by the size of the inoculum, at the temperature of 41° C. At the optimum temperature the final increase in amino nitrogen does not seem to be dependent upon the size of the inoculum, within the limits tested.

Reports on the influence of the size of the inoculum upon bacterial growth are frequent in the literature. Many of these are apparently limited to the ability to initiate growth, as in the case of Cole's experiments with the pneumococcus.* Studies on the rôle of accessory food substances in bacterial growth and nutrition furnish examples of similar phenomena, together with at least a partial explanation of many of the earlier observations.* With these reports the present paper is not concerned.

Here, it is merely desired to point out that at temperatures above the optimum, the luxuriant growth of microörganisms may exhibit much greater dependence upon the size of the inoculum, than at optimum temperatures. This, of course, is to be expected but the factors involved should not be discussed without a greater amount of experimental evidence.

In a number of our 41° tests, the influence of the size of the inoculum does not seem to be limited to the ability to initiate growth. Frequently, small inocula gave slight growth but never approached the growth evident in tests of the same series with large inocula. This obtained even when cultures were incubated until absolutely sterile.

* Personal communication of unpublished work, by Dr. O.T. Avery.

4. Influence of temperatures above the optimum upon the final H-ion concentration in glucose broth.

In several tests made in conjunction with the preceding experiments, the ability of the different strains to grow at 41° C. was controlled by culture in glucose broth. In one of these tests the pH of a glucose broth culture of the lactic strain was roughly tested by the addition of several drops of methyl red. This culture gave a salmon yellow color, which indicated that lower final H-ion concentrations were attained at temperatures above the optimum.

It seemed desirable to study the influence of such temperatures upon the final H-ion concentrations reached by the different type strains. The results obtained would be of value in the interpretation of the general influence of high temperatures upon the life processes of the different streptococci. They would also represent a small contribution to our knowledge of the various environmental factors which may at times obscure the specific effect of the H-ion.

Procedure: (Experiment 8)

Medium: Glucose infusion broth. 50 cc. portions of the medium were brought to a temperature of 41° C. They were then inoculated with 0.3 cc. of a 12-hour culture of the respective strains. The inoculated flasks were placed in the 41° water bath used in the previous experiment.

Another series received the same inocula and was incubated at 37° C.

After 5 days incubation electrometric determinations of the final pH were made by Dr. A. Itano. It is believed that these figures represent final values as all of the 41° test cultures were sterile at the time of pH determinations with exception of the cheese strain. This culture contained comparatively few viable cells by the plate method.

The results are given in Table X.

Table X.

Comparison of Final H-Ion Concentrations of

Glucose Broth Cultures at 37° and at 41° C.

Medium: glucose infusion broth, p H 7.4. Inocula: 0.5 cc. of 12-hour cultures, in 50 cc. of test medium. Cultures 5 days old; human, bovine, and lactic cultures of the 41° series were sterile at the time of pH determinations.

	Human	Bovine	Lactic	Cheese
41°	5.6	4.7	5.0	4.3
37°	5.0	4.4	4.2	4.1

The results in Table X are believed to represent final values with the possible exception of the cheese strain. The fact that the human, bovine, and lactic cultures were sterile at the time of the test, requires that any further acid production be due to purely enzymatic reactions. The well known sensitivity of lactic acid producing enzymes renders it improbable that these enzymes of the streptococci are more resistant to exposure to high H-ion concentrations at 41°, than are the cells themselves. The work of Avery and Cullen on the enzymes of pneumococcus (a close relative of the streptococci) has shown that its enzymes are relatively sensitive to deleterious influences.

If these figures are final values, and there is little reason to believe they are not, the final H-ion concentration reached in glucose broth at temperatures above the optimum are significantly different than those obtained at the usual incubation temperatures. The difference is especially evident in the case of the human and lactic strains which throughout our study have proven relatively more susceptible to high incubation temperatures than have the bovine and cheese strains. There is nothing surprising in the above findings, altho' similar examples in case of streptococci have not been given in the literature. It is especially interesting, to note that the lactic strain would be "methyl red negative" at this temperature.

The importance of the above results to the relation of H-ion concentration to general microbiology is as further evidence that other factors often obscure the specific effect of the H-ion. Recent literature has offered numbers of examples in support of Clark's early contention that the "physiological constant" conception of the final H-concentration requires a strict definition of the fermentation system.

The lower "fermentation limit" reached at temperatures above the optimum may be explained most easily by the

$$\text{Speed} = \frac{\text{Driving Force}}{\text{Resistance}}$$

formula used by Getman to explain the end points or final equilibria of cayalytic reactions. It is only reasonable to suppose that the "resistance" at 41° is greater than at the optimum temperature. Hence, the product of all of the factors involved in the final inhibition of acid production, would reach a value reducing the "speed" to zero at a H-ion concentration which in itself would be insufficient to prohibit further acid production at the optimum temperature. Again, a positive temperature coefficient for the product of all of those forces which are involved in the final inhibition of growth of bacterial cultures, would also require a lower final acidity at a higher temperature.

The factors conditioning the "fermentation limit" of microorganisms are discussed in detail in Part I of this paper. (See "Influence of H-ion Concentration" under " Influence of the Environment of Lactic Acid Bacteria"; also, "End Point of the Lactic Acid Fermentation Process" under "Theoretical Progress of Lactic Acid Fermentation".)

Fairly large inocula were used in the above tests. Whether the size of the inoculum also influences the final pH at 41°, is not known. A study of this factor was not made as the direct object of this experiment was merely the comparison of the relative influence of higher incubation temperatures upon the life processes of the different types of streptococci.

5. Influence of temperature upon the rate of growth and upon the activity and viability of different types of streptococci.

The influence of temperature upon the products of microbial activity has been studied in the preceding experiments. On last analysis, the influence of temperature upon the accumulation of the products of microbial processes is in many cases, at least in part, a reflection of the influence of those temperatures upon the sum total of all of those processes which are involved in the actual growth of the cells. The relation, however, is not necessarily parallel, nor always direct. The accumulation of certain products is not due entirely to the actual growth of the cells, as was suggested by the results obtained in Experiment 3 (Table VI).

For the above reasons it was desired to compare the direct influence of different temperatures upon the growth and multiplication of the different streptococci. It was also desired to compare the general vitality of cultures of the different types, which had been incubated at different temperatures for equal periods of time. The continuation of such a comparison to longer periods of exposure offered a means of comparing the relative longevity of the different types after equal time exposures to different temperatures. The following experiment was conducted in the study of these relations.

Procedure: (Experiment 9).

The experiment has the following direct objects:

(1) An approximate comparison of the relative rate of growth of the different types of streptococci at different temperatures.

(2) A comparison of the "general growth condition" and vitality of cultures of equal age which have been growing at different temperatures; this extends itself to a comparison of the relative longevity of the different streptococci after equal time exposures to different temperatures.

a. (Object I.)

Relative rate of growth of the different streptococci at different temperatures.

The satisfaction of Object I required an approximation of the relative rate of growth in cultures incubated at different temperatures. The procedure employed to meet this end involved the estimation of the relative number of cells present in the various members of a series of broth cultures which had received equal inocula of the respective strains and which had then been incubated for an equal period of time. This set of conditions suggested the use of the method described as the "Andrade test" in the statement of "Methods of Study". In the approximate comparison of numbers, this method has been limited to tests made with young cultures; it also requires that no member of the test series has been exposed to previous unfavorable environments. This set of conditions will obtain in young cultures of the 15^{0}, 23^{0}, and 32^{0}, members of the temperature series. It is not met, however, in the 41^{0} test as there is a strong probability that inocula taken even from young cultures grown at temperatures above the optimum cannot be compared with those taken from cultures held at lower temperatures.

b. (Object II.)

Comparison of the relative activity and vitality of cultures of the different types of streptococci when incubated at different temperatures.

The satisfaction of Object II requires an approximation of the "general growth condition" or vitality of the different cultures. The principles involved in such an approximation have been developed in the description of the "Andrade test". ("General Methods of Study"). From the relations discussed in that place, it is evident that the conditions underlying

the satisfaction of Object II are met by the second set of conditions described in our former explanation of the above method.

The relative rate of acid production of inocula taken from these cultures will be an approximate function of the relative growth condition and vitality of the various cultures under comparison. Here, one is not limited to the comparison of young cultures nor to temperatures not above the optimum. In fact, the actual object in mind is the relative comparison of the influence of all of the various conditions in the environments obtaining at different temperatures. For this reason the use of the above described method seems to be justified, in an approximate estimation of the relative influence of different temperatures upon the vitality of the different types of streptococci.

Object II also requires the comparison of the relative longevity of the cultures at different temperatures. The above method, of course, is essentially a test of the presence of viable cells. However, for actual determinations of sterility larger samples must be taken as test inocula.

Detailed procedure:

The temperature series were prepared as described in Experiment 5; equal volumes of broth received equal inocula of the respective type strains; these cultures were incubated at the temperatures of 15°, 23°, 31°, and 41°, respectively. (The test media were maintained at the proper temperatures previous to, and during inoculation.)

The detailed manipulation involved in the "Andrade test" has been given in the description of the method. (General Methods of Study). One cc. samples were removed from each member of the series; the sample was diluted in sterile salt solution; 1.0 cc. of the dilution (representing 0.01 cc. of the original sample) was introduced into uniform test tubes containing 12 cc. of sterile glucose Andrade infusion broth. These tests were incubated at 37° C., and observations made of the time required for the attainment of a distinct pink color. Tests were made in duplicate. The 1 day test period was used in the approximation of the rate of growth. The 3, 5, and 15 day tests were used in the comparison of the general growth condition and vitality of the cultures.

For a check on the possible sterility of cultures, undiluted 1.0 cc. samples were introduced into glucose broth. This test was made at each of the time periods in the case of the 41° series, and at the 15 day period with all of the cultures.

The data obtained are given below in Table XI; they are restated in Tables XII and XIII.

Table XI.

Data Obtained in Experiment 9.

Hours Required for Acid Production by Inocula Taken from Cultures Incubated at Different Temperatures.

Temperature test series received equal inocula of cultures of each type. These cultures were incubated at different temperatures. Figures below represent time required for production of equal amounts of acid by equal samples taken from members of the above temperature series after stated periods of incubation.

	Age of Culture. days	15°	23°	32°	41°
Lactic					
	1	9	6¾	6¼	9
	3	7½	7¾	8	36
	5	8	8	8½	sterile
	15	14¼	16	18	-
Human					
	1	19	11	10¼	32
	3	16	7¾	8½	sterile#
	5	18	11	12¼	sterile
	15	24-36	24-36	24-36	-
Bovine					
	1	13	7½	6¾	9½
	3	12¾	6	6½	11
	5	13	6½	7¼	13
	15	14	9¾	10½	sterile
Cheese					
	1	10	6	6	6
	3	7½	6	6	6
	5	6½	6½	7	8½
	15	7¼	8¼	10	12¾

.01 cc. sterile; 1 cc. positive in sterility tests made in glucose broth.

The data reported as a whole in Table XI can be used to better advantage in the interpretation of the problems in mind by an independent attack upon each of the objects of the experiment.

a. (Object I.)
Relative rate of growth of the different streptococci at different temperatures.

The use of the data in Table XII in this connection is limited. For reasons stated above, none of the figures obtained in the 41° tests can be interpreted here. Altho' 24-hour broth cultures of streptococci are not young cultures when incubated at the usual incubation temperatures, there should be no serious objection to the use of cultures of this age which have been grown at the lower temperatures of this series.

Hence, it seems that these figures should serve for an approximate comparison of the relative influence of different temperatures upon the rate of growth of the different type strains. With this interpretation the 24-hour values reported in Table XII are restated below.

Table XII.

Influence of Different Temperatures Upon the Rate of Growth of Different Types of Streptococci.

Relative rate of growth at different temperatures, based upon the comparative rate of acid production of equal inocula taken from cultures which had been incubated 24 hours at different temperatures.

In the table below are presented the reciprocals of the 15°, 23°, and 32° values given in Table XI--i. e., these figures represent the reciprocals of the number of hours required for the production of equal amounts of acid by equal inocula of cultures which had been incubated 24 hrs. at the stated temperatures. These reciprocal values do not have a common basis, and are related only to the values obtained for the same strains.

	15°	23°	32°
Lactic	.11	.15	.16
Human	.05	.09	.10
Bovine	.07	.15	.15
Cheese	.10	.17	.17

The following general relations are evident in Table XII.

The lactic streptococcus exhibited its most rapid growth in the temperature series approximating 30°. Its rate of growth at 15° is relatively greater than that of any of the other types. The human and bovine strains also show more rapid growth at 30° than at lower temperatures. At 41° the growth of the human strain is inhibited to a more marked degree than is that of the mastitis strain.

The figures obtained for the cheese strain are of little meaning in the direct interpretation of the influence of temperature upon the rate of growth. Apparently, the cheese type had reached its maximum growth in 24 hours at all temperatures except the lowest member of the series. With the resistance peculiar to this strain, the activity of the cultures at these temperatures did not rapidly diminish after the attainment of maximum growth.

b. (Object II.)

Comparison of the relative activity and vitality of cultures of the different types of streptococci when incubated at different temperatures.

For reasons given above in the preceding discussion, it seems that all of the data presented in Table XI can be used in comparing the relative influence of different temperatures upon the general activity and vitality of the different streptococci. To serve as a means of more ready comparison of the figures obtained, they are presented in a slightly different form in Table XII.

In the case of each strain, the test which showed the most rapid acid production is assumed to represent the temperature-time period in which that strain exhibited its greatest activity. This assumption is of course limited to the temperature-time periods tested. A larger number of tests would be required for the "period of greatest activity" so obtained, to represent anything approaching an absolute value.

In the presentation below, (Table XIII), the temperature-time "period of greatest activity" of the different strains is expressed as unity. The reciprocals of the time required in the other tests of each strain is then multiplied by the time required in the test taken from the temperature-time "period of greatest activity" of the same strain. The relation between these figures is by no means absolute or definite. Nevertheless, there is reason to believe that they may serve as convenient indices of the influence of different temperatures upon the general activity and vitality of the different streptococci. These figures are given in the following table.. (Table XIII).

Table XIII.

Relative Activity and Vitality of Cultures of Different Types of Streptococci Grown at Different Temperatures.

(See text for limitation of values reported below, and for the basis of their assignment).

	Age of Culture. days	15°	23°	32°	41°
Lactic	1	.69	.93	1.00	.69
	3	.83	.81	.78	.18
	5	.78	.78	.74	---
	15	.44	.39	.35	---
Human	1	.41	.70	.76	.26
	3	.49	1.00	.92	---
	5	.43	.70	.64	---
Bovine	1	.46	.80	.89	.63
	3	.47	1.00	.92	.55
	5	.46	.92	.83	.43
	15	.43	.62	.57	---
Cheese	1	.60	1.00	1.00	1.00
	3	.80	1.00	1.00	1.00
	5	.92	.92	.86	.71
	15	.83	.73	.60	.47

The values given in Table XIII show the following general relations:

Growth of the lactic strain is apparently inhibited to a less extent by low temperatures than is that of the other type strains. The human strain exhibited the narrowest range of temperature for growth.

Both the lactic and the human streptococci, while able to grow to some extent at 41° C., are rapidly weakened and killed after comparatively short exposure to that temperature. As in previous experiments at 41°, the life processes of the cheese and mastitis strains are inhibited to a less extent.

The resistance of the cheese strain at this temperature was striking as a survival of 15 days at 41° C. is a surprising exhibition for a hemolytic streptococcus. However, this strain also proved exceedingly resistant to all other unfavorable environmental conditions. On the other hand, the relative resistance of the mastitis strain at 41° is more interesting. Its resistance relative to the human strain is easily explained by assuming the human streptococcus to be in general a more delicate organism. However, the greater resistance of the mastitis strain in comparison to the lactic streptococcus cannot be explained upon a similar basis.

It was found that the bovine strain was less resistant in heating experiments conducted at temperatures above 60° C. (In tests made in milk, the lactic survived the conditions of the pasteurization process, while the mastitis strain quickly succumbed.) While these two experiments are not directly comparable, it is evident that the mastitis streptococcus is not more resistant to a given period of exposure to temperatures above 60° C. Such a relation might point to a possibility of differences in the temperature coefficients of the disinfection rates of these two strains between 41° and 63° C. The question

however, is obscured by the probable influence of previous culture upon the resistance of both strains to heat. In the absence of further experimental data, it is more wise to assume that the difference in longevity of the bovine and lactic strains at 41° C., represents a relation dependent upon the fact that this temperature is further removed from the optimum temperature for growth of the lactic strains.

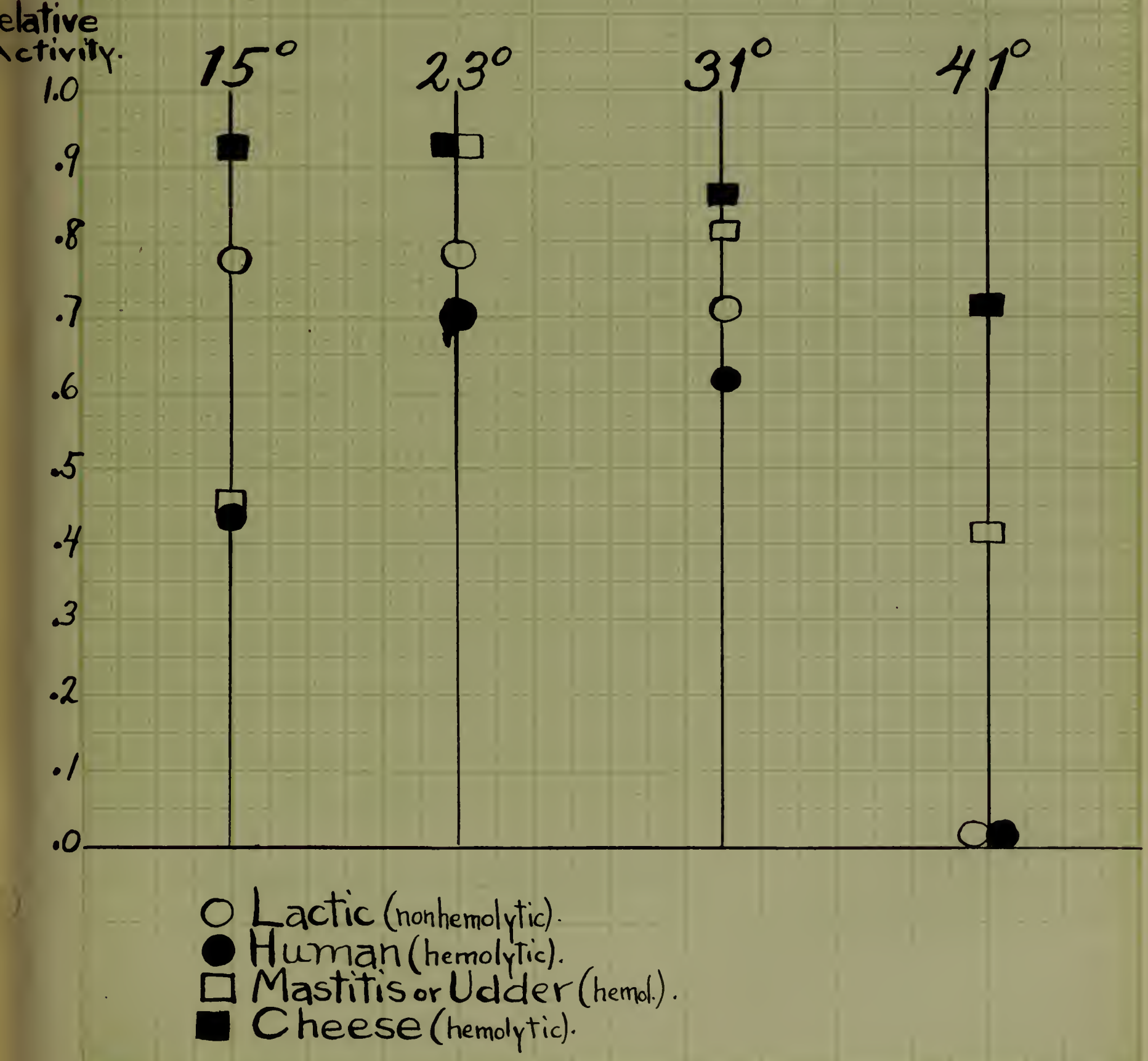
The relative activity of cultures which, after receiving equal inocula of each type strain, were incubated for 5 days, may be used to illustrate the general behavior of the different type strains in environments of different temperatures. These 5 day values are plotted below in Figure 2. This figure is well adapted to an immediate survey of the general relations of the type strains to the different test temperatures.

The following relations are among those evident in Figure 2. The 15° values for the human and bovine strains represent cultures which have not multiplied since the initial inoculum (compare values given in Table XIII). The cheese and lactic strains, which grew at this temperature, have diminished in activity to a less extent at 15° than at the higher temperatures. The well known greater longevity and more persistent activity of cultures held at lower temperatures is evident in all of the series in the figure, and requires no further discussion. The sudden drop in activity of the 41° tests of the human and lactic strains is indicative of the sensitivity of these strains to temperatures above 37° C.

Figure 2.

Relative Activity of Cultures of Different Types of Streptococci, which Have Been Incubated 5 Days at Different Temperatures.

(values plotted below are taken from Table XIII.)



6. Summary and discussion of the influence of temperature upon the life processes of the different streptococci.

Influence upon amino nitrogen increases.

The lactic strain exhibits greater relative activity at temperatures below 30° than do any of the other type strains. The lactic streptococcus produced a higher final concentration of amino compounds at a temperature lower than that required for greatest rate of formation of amino compounds. This is not an unusual observation as frequently higher final end points are attained at temperatures below that at which the greatest velocity is displayed. (See "Theoretical Progress of Lactic Acid Fermentation" - Part I of this paper).

At 41° C., the peptone splitting powers of the bovine and the cheese strains are inhibited to a less extent than are those of the lactic and the human strains.

Influence upon final H-ion concentration.

At 41° C. lower final true acidities are reached in glucose broth by the different type strains than at 37°. As in the case of the amino nitrogen production, the inhibition of the bovine and cheese strains is relatively less than that of the human and lactic types.

Influence upon growth and activity.

Much the same is the influence of temperature upon the rate of growth as upon the rate of amino production. As is to be expected, the decrease in activity is less at lower temperatures than at high temperatures in the case of all strains. The bovine

and cheese strains not only grow more actively at 41° than do the other types, but also decrease in activity less rapidly at this temperature.

In the case of the bovine strain, it does not seem that its relative resistance to this temperature is due to an intrinsic resistance to disinfection by heat. It seems more probable that 41° merely represents a point nearer the optimum temperature of the bovine than of the lactic strain, rather than that the bovine streptococcus is more resistant to a given period of exposure to high temperatures.

Practical significance.

The practical significance of these temperature relations consists in the relation of temperature to the determination of the relative number of different types of streptococci present in milk and milk products held at different temperatures. The human and mastitis strains (in addition to the before mentioned influence of H-ion concentration) would be outgrown in milk held at low temperatures by the lactic and cheese strains.

It is also evident that both the cheese and lactic strains could attack the nitrogenous constituents of cheese at low temperatures.

IV. Influence of Oxygen Concentration upon Amino Nitrogen Increases.

The influence of oxygen concentration as a factor in the environment of lactic acid bacteria has been reviewed in detail in Part I of this paper. ("Influence of Environment upon Lactic Acid Bacteria".) There it was shown that this factor plays an

important rôle in th. determination of many microbial processes. The importance of oxidation processes as a means of obtaining energy for microbial growth has also been discussed in detail in Part I. ("Chemical Changes Involved in Lactic Acid Fermentation"- "Transformation of Energy".) The general significance of an investigation of the influence of oxygen concentration upon the activity of the different types of streptococci is evident by a review of those discussions.

The direct significance of a comparison of the influence of different oxygen concentrations upon the peptone attack of the different streptococci, lies in the fact that these different types of streptococci frequently are found at different times in systems of widely different oxygen concentrations. The "human" and "bovine" streptococci are commonly found in the animal body under conditions, possibly differing in oxygen concentration. Lactic streptococci are also found under wide ranges of oxygen concentration. The concentration of oxygen in milk is rapidly changing during microbial growth, and as Marshall* has shown, soon approaches a minimum. In cheese and other systems in which the lactic group are involved, the concentration of oxygen may also be a factor in determining the rate, the extent, or possibly the direction of the changes brought about by lactic acid bacteria.

The importance of all of these relations pointed to the pertinence of the study of the relation of oxygen concentration to the peptone attack of the different types of streptococci. The investigation of these relations consisted in the comparison

*Personal communication.

of the amino nitrogen increases effected by the different streptococci, when grown in systems differing only in the concentration of atmospheric gases.

Procedure: (Experiment 10).

Anaerobic conditions were obtained by the method suggested by the recent work of Bates and Olitsky. The principles of the method consist in deoxygenation of the medium by boiling and overlaying the deoxygenated medium with sterile melted vaseline. The melted vaseline serves as an effective seal inhibiting the access of oxygen. Anaerobic conditions are maintained by the reducing action of peptone in faintly alkaline systems at a temperature of 37° C. Methylene blue was used as the indicator of the effectiveness of the removal of oxygen and of the maintenance of deoxygenation.

Detailed procedure: 50 cc. of infusion broth, pH 7.3, was sterilized in the tubes commonly used in the "reductase" test in milk analysis. 0.4 cc. of a 1 per cent sterile solution of methylene blue was added to one tube to serve as a later check on the anaerobic conditions. The tubes and check were placed in the steamer at 100° C. and boiled until decolorization of the check indicated that the dissolved air was driven out of the medium. The tubes were then removed separately, and the medium overlayed with sterile melted vaseline and immediately cooled in running water to solidify the vaseline. The same manipulation was employed with the check. The methylene blue remained decolorized during the time required for the overlaying of the vaseline, which proved that appreciable amounts of oxygen did not enter the medium during this procedure. The tests and the check were then incubated for 72 hours at 37° C. to ensure sterility and anaerobic conditions.

The same broth was used in the case of the aerobic series. In this case, 50 cc. portions were sterilized in Erlenmeyer flasks and subjected to the same heating conditions as the anaerobic series.

The two series received equal inocula of each strain. The anaerobic series were inoculated by means of a capillary pipette. The lactic tests were incubated at 32° C; the other strains, at 37° C. After 8 days incubation, NH2-N determinations were made. Results are given in Table XV.

Table XV.

Influence of Oxygen Concentration upon Increases in Amino Nitrogen.

Medium: Infusion broth, pH 7.3. Anaerobic conditions were maintained by vaseline seals. Cultures 8 days old at time of analysis. Results are expressed as mg. NH_2-N per 100 cc.

	Aerobic		Anaerobic	
	Total	Increases	Total	Increases
Lactic	55.8	4.8	56.2	5.2
Human	60.8	9.8	60.7	9.7
Bovine	55.6	4.6	55.8	4.8
Cheese	60.6	9.6	60.1	9.1
Control	51.0	---	51.0	---

The results given in Table XV show that the human, bovine, and lactic strains were not greatly influenced by the concentrations of oxygen tested in the above experiment. This is quite in accordance with the usual assumption that most streptococci are "facultative anaerobes". The cheese strain alone appears to attack peptone with greater avidity under aerobic conditions.

V. Optimal Environmental Conditions for the Different Types as Shown by the Foregoing Study.

The experiments just reported offer a means of choosing the environmental conditions under which to conduct the comparison of the peptolytic activity of the different types of streptococci to be studied in Section B. The following summary presents the conditions chosen as representing optimal conditions for the different streptococci.

H-ion concentration zones.

Lactic and cheese strains - pH 6.5

Bovine and human strains - pH 7.5

Temperature.

Lactic strains - approximately 30° C.

Other strains - 37° C.

Oxygen Concentration.

Little difference was manifested between aerobic and anaerobic cultures of the different streptococci. For purpose of convenience aerobic cultures will be used in Section B.

These conditions will be maintained in the investigation reported in Section B.

Section B.

COMPARISON OF THE PEPTOLYTIC ACTIVITY OF DIFFERENT TYPES OF STREPTOCOCCI.

I. Preliminary Statements.

1. Relation to preceding studies.

The preceding studies have been concerned primarily with the establishment of definite optimal environmental conditions for the peptolytic action of the different streptococci. The short summary given on the preceding page states the conditions established as optimum for the different types. The following studies are concerned with measurements of the amino and ammonia compounds which are formed by a larger number of strains of the various types when growing in peptone broth under the foregoing standardized optimum environmental conditions.

Such measurements of the relative production of these products by a number of strains of different types should furnish a certain contribution to our knowledge of differences or likenesses in metabolism between different groups of streptococci. At the same time, they offer a similar means of comparison between different members of the same type of streptococci, as now recognized in present systems of grouping these organisms.

The value and worth of such comparisons is largely conditioned by the number of strains included in the tests. However, they are conditioned to an equal extent by the knowledge of other characters, and of the previous history of the strains which are studied. It is obvious that a comparison of the peptone splitting activity of the human and lactic groups of streptococci requires some assurance that the strains upon which the comparison is based possess the characters usually assigned to those groups. The number of strains included in the following tests is not large. However, it is believed that they represent a fairly typical collection of the different types. The following description of the strains is added to that furnished in the introduction.

2. Descriptions of groups and strains studied:

Lactic group:

Strains from sour milk and fermented dairy products are included in this collection. In Part II of this paper, these strains are completely described, at which place evidence is presented that most of these strains would probably be included in the so-called Strep. lacticus group. Five strains (Sk, S, C, W, G) agree in all characters with Evans' characterization of this group. The remaining strains, with the possible exception of strain X, agree with the usual characterization of the lactic type. All of them reduce litmus before coagulation in litmus milk cultures, actively acidify milk, grow at low temperatures, and produce H-ion concentrations more acid than pH 5.0 in glucose broth. With the exception of strain X, none of the sour milk strains are hemolytic on blood agar. Strain X represents a hemolytic sour milk strain, which, to say the least, is closely related to the typical lactic streptococci. (Strain PD, the other hemolytic sour milk strain described in Part II, was lost through a laboratory accident.)

The first mentioned five strains are termed "lactic" streptococci with apparent justification; the remaining strains are termed "non-hemolytic sour milk" and "hemolytic sour milk" strains. It is probable, however, that all of the non-hemolytic strains possess a sufficient number of characters in common to be included in the large lactic group.

Hemolytic human group:

This group is represented by eleven strains which were furnished by Dr. O. T. Avery as a typical collection of hemolytic streptococci from human pathological conditions. These strains agree in the

following characters: Exhibit final H-ion concentrations approximately pH 5.0, do not grow at 10° C., and are associated with human pathological conditions. These strains have been further described in Part II of this paper, and by Avery and Cullen (1919), and by Dochez, Avery and Lancefield. The actual source of these strains is given in Table XVII.

Hemolytic mastitis or udder group:

This group is represented by eight strains which were furnished by Dr. O. T. Avery. These strains include hemolytic strains obtained from the udder and from cases of mastitis. They represent members of the "bovine" group of hemolytic streptococci. Because of the probable heterogenous character of the "bovine" or "high-acid" group of hemolytic streptococci, our comparison is limited to its members obtained from the udders of cows. These strains differ from the preceding group in the exhibition of H-ion concentrations more acid than pH 5.0. None of these strains are able to grow at 10° C. in glucose broth.

Hemolytic cheese group:

This group is represented by only one strain, "Gun.", which was also used as a type strain in the preceding studies. This group has not been studied to so great an extent as the preceding groups, but a complete description of the group may be found in the recent work of H. C. Avery. The description of this type strain has been given previously (Tabular Summary, Part II).

Non-hemolytic sauerkraut strain:

The non-hemolytic sauerkraut strain described in part II has been included in a part of this study. It is not presented as a typical representative of any particular group, as comparatively little is known of the streptococci of fermented plant products. This strain is described quite completely in the Tabular Summary in Part II.

II. Comparison of Amino and of Ammonia Nitrogen Increases Exhibited by Different Members of the Lactic Group, and by Strains of Other Types of Streptococci.

It is desired to compare the increases in amino and ammonia nitrogen brought about in peptone broth by different members of the lactic group. It is further desired to compare these increases with those effected by other types of streptococci when grown under similar conditions. The attainment of these objects presents a means of showing differences and likenesses in this phase of the metabolism of different lactic streptococci, and of comparing the products accumulated in similar cultures of different types of streptococci.

The tests have been made in broths of a pH value approximating that of fresh milk. This represents a H-ion concentration zone more favorable to the lactic and cheese strains than to the human and mastitis strains. Optimum temperature conditions were maintained for the various types.

Procedure:

Infusion broth, pH 6.6, containing 0.2 per cent sodium phosphate, was inoculated with the seventeen lactic or non-hemolytic sour milk strains, the hemolytic sour milk strain, a representative strain of both the hemolytic human and hemolytic mastitis collections, the hemolytic cheese strain, and the non-hemolytic sauerkraut strain. The sour milk strains and the sauerkraut strain were incubated at 31° C., the other type strains at 37° C. Amino and ammonia nitrogen determinations were made after 12 days incubation. Results are given below in Table XVI.

Table XVI.

Amino and Ammonia Nitrogen Increases Effected by Different Members of the Lactic Group, and by Strains of Other Types of Streptococci.

Medium: infusion broth, pH 6.6. All of the sour milk strains were incubated at 31° C.; other type strains, at 37° C. Cultures were 12 days old at the time of analysis. Results given below as mg./100cc.

		Total NH_2-N	Total NH_3-N	Increase in NH_2-N	Increase in NH_3-N
	Lactic strains				
SK	(non-hemolytic)	61.2	16.4	4.5	7.7
S	" " "	61.4	16.7	4.5	8.0
C	" " "	62.4	15.6	5.5	6.9
W	" " "	61.6	15.1	4.7	6.4
G	" " "	61.9	16.1	5.0	7.4
	Non-hemolytic sour				
IF	milk strains	60.7	----	3.8	---
M	" " "	59.4	----	2.5	---
MAC	" " "	58.5	11.6	1.6	2.9
Z	" " "	61.5	----	4.4	---
1	" " "	61.0	17.1	4.1	8.4
2	" " "	61.8	----	4.9	---
3	" " "	60.0	----	3.1	---
4	" " "	60.0	----	6.1	---
5	" " "	61.7	----	4.8	---
6	" " "	60.4	----	3.5	---
7	" " "	61.0	----	4.1	---
8	" " "	63.2	----	6.3	---
X	Hemolytic sour milk strain	62.6	15.7	5.7	7.0
S32	Hemolytic human strain	64.4	17.0	7.5	8.3
C67	Hemolytic mastitis strain	60.7	16.2	3.8	7.5
MAN	Hemolytic cheese strain	67.5	17.4	10.6	8.7
K	Non-hemolytic sauerkraut strain	56.8	10.0	----*	1.3
Control		56.9	8.7	----	---

*9-week old culture of same strain in same broth gave increase of 0.9 mg. NH_3-N.

The results given in Table XVI show the following general relations:

The lactic and non-hemolytic sour milk strains show considerable variations in the amount of amino and ammonia compounds formed in peptone broth. The increases in amino nitrogen varied from 1.6 to 6.3 mg. per 100 cc. The increases in ammonia nitrogen varied from 2.9 to 8.0 mg. per 100 cc. Although the strain which exhibited the lowest final increase in amino nitrogen also produced the smallest increase in ammonia, there seems to be no definite relation between the increases in these two products by the various strains. Not infrequently a strain which produces a greater increase in ammonia than another strain, shows a smaller increase in amino acids.

In this medium, the hemolytic sour milk strain shows an amino nitrogen increase slightly higher than the average among the non-hemolytic strains. It does not, however, show a greater increase than that exhibited by several other sour milk strains, in this pH zone.

The hemolytic mastitis strain shows an amino nitrogen increase within the range exhibited by the lactic sour milk group. On the other hand, both the cheese and human hemolytic strains produce greater increases in amino acids than do any of the members of the lactic group. It must be remembered, of course, that the pH of this medium is more favorable to the lactics than to the mastitis and human strains.

The sauerkraut strain at the end of 12 days incubation, showed no increase in amino nitrogen. A nine week old culture of the same strain showed a small but significant increase of

0.9 mg. This finding is in accord with the discussion of the meaning of increases in amino compounds, which has been given in Section A. (" Influence of the Stage of Growth of the Culture upon Increases in Ammonia and Amino Nitrogen".)

III. Comparison of Amino Nitrogen Increases Effected by Different Types of Streptococci.

The preceding experiment has shown that the final increases in amino nitrogen seemed to differ in the case of certain of the different types of streptococci. A greater divergence was shown to exist between the increases in amino nitrogen manifested by the different types, than between their increases in ammonia. For this reason, the present experiment was limited to comparisons of the production of amino compounds.

The primary object of the experiment is the comparison of the amino nitrogen increases brought about by different types of streptococci. The inclusion of a larger number of strains of the human and mastitis types was essential for this purpose. The study of a number of strains of these types also offered a means of comparing the amino nitrogen increases manifested by different members of the respective groups.

In this experiment, a H-ion concentration is chosen which approximates that of blood, and of the usual bacteriological media. This represents the pH zone more favorable to the human and bovine streptococci. Two strains of lactic streptococci are included as typical representatives of the lactic group. The hemolytic sour

milk strain is also included in an attempt to compare its relation to the other types, in a pH zone usually more favorable to hemolytic streptococci than that used in the preceding experiment. The hemolytic cheese strain is included for comparative purposes.

Procedure: (Experiment 12).

Infusion broth, pH 7.3, containing 0.2 per cent sodium phosphate, was inoculated with each strain of the collection of human and mastitis streptococci, and with two representative lactic strains, the hemolytic sour milk strain, and the hemolytic cheese strain. After 10 days incubation at 37° C., amino nitrogen determinations were made.

Results are given in Tables XVII and XVIII.

Table XVII.

Amino Nitrogen Increases by Streptococci from Different Sources.

Medium: Infusion broth, pH 7.3, containing 0.2 per cent sodium phosphate. Cultures incubated ten days at 37° C. before analysis. Results given as mg. /100 cc.

Strain	Source	Amino Nitrogen Total	Amino Nitrogen Increase
S23	Throat	59.3	7.6
S125	Throat, lobar pneumonia	61.3	9.6
S72	Throat, lobar pneumonia	61.1	9.4
S55	Sputum, broncho pneumonia	59.5	7.8
S67	Blood, broncho pneumonia	60.6	8.9
S271	Septicemia	62.7	11.0
S3	Lung autopsy, broncho pneumonia	58.3	6.6
S32	Lung autopsy, broncho pneumonia	60.9	9.2
S84	Pleural fluid	59.8	8.1
S273	Scarlet fever	61.5	9.8
S70		59.7	8.0
V1	Udder	56.4	4.7
V2	Udder	57.0	5.3
C53	Mastitis	57.4	5.7
C57	Mastitis	58.2	6.5
C59	Mastitis	55.8	4.1
C67	Mastitis	57.6	5.9
C69	Mastitis	57.4	5.7
M26	Mastitis	58.3	6.6
S	Sour milk (non-hemolytic)	55.7	4.0
G	Sour milk "	56.2	4.5
X	Sour milk (hemolytic)	59.7	8.0
MAN	Cheese (hemolytic)	60.6	8.9
Control		51.7	---

Table XVIII.

Amino Nitrogen Increases by Different Types of Streptococci.

Results presented in Table XVII are rearranged below, with strains grouped according to types. Figures given below represent mg. NH_2-N/100 cc.

Hemolytic Mastitis or Udder Strains		Hemolytic Human Strains	
V1	4.7	S23	7.6
V2	5.3	S125	9.6
C53	5.7	S72	9.4
C57	6.5	S55	7.8
C59	4.1	S67	8.9
C67	5.9	S271	11.0
C69	5.7	S3	6.6
M26	6.6	S32	9.2
		S84	8.1
		S273	9.8
		S70	8.0

Non-hemolytic Lactic or Sour Milk Strains.		Hemolytic Sour Milk Strain	
S	4.0	X	8.0
G	4.5	Hemolytic Cheese Strain	
		MAM	8.9

In Table XVIII, the variations in amino nitrogen production by different members of the human and the bovine hemolytic groups are seen to cover about as wide a range as that exhibited by the different non-hemolytic sour milk or lactic strains in the preceding experiment. In the pH zone of the last experiment, the mastitis collection effected approximately the same range of amino nitrogen increases as that shown by the sour milk or lactic group in the pH 6.6 zone.

In spite of the variation within the different groups, a certain divergence in the amino acid production is evident between the different types. The minimum increases effected by the human collection is no less than the maximum effected by any of the eight bovine strains studied. Of course, there is no reason to believe that a study of a larger number of strains of both types might not reveal a considerable overlapping of the range of amino acid production of the two types.

If the hemolytic sour milk strain is to be considered a member of the so-called lactic group, an example of such an overlapping of increases in amino nitrogen is presented above. However, it is also evident that the amino acid production of this strain approximates that of the representative of H. C. Avery's group of hemolytic cheese streptococci.

The variations within the different groups and the divergence in amino nitrogen increases by the different types are more evident in Figure 3.

Amino Nitrogen Increases by Different Types of Streptococci.

(values given below for Lactic strains are from Table XVI; other values, from Table XVII.)

Fig. 3

TYPE.

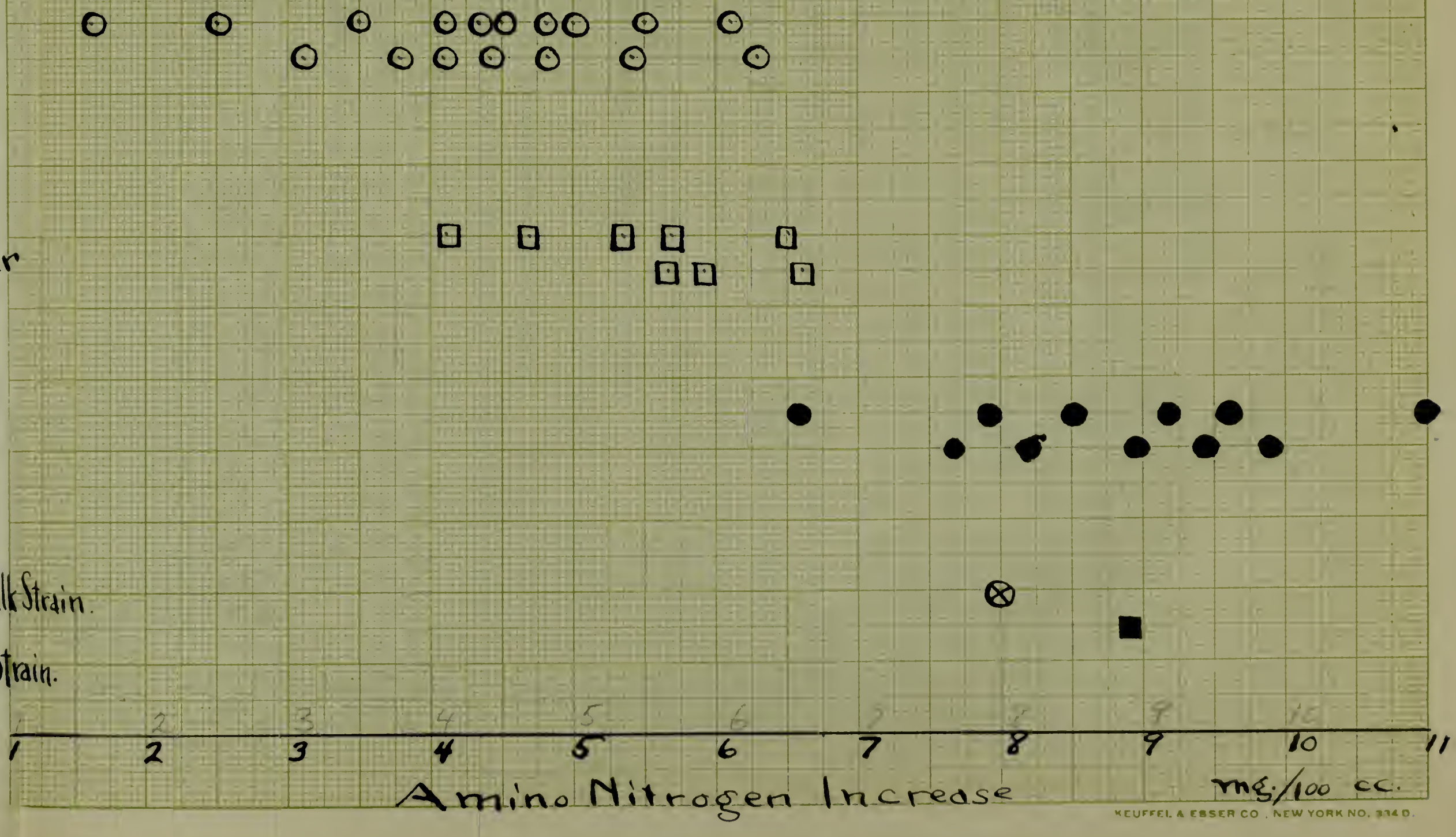
Lactic (non-hemolytic)

Mastitis or Udder (hemolytic)

Human (hemolytic)

Hemolytic Sour Milk Strain.

Hemolytic Cheese Strain.



IV. General Discussion of the Comparative Peptolytic Activity of Different Types of Streptococci.

Increases in Amino Nitrogen.

The collections of lactic or non-hemolytic sour milk strains, the hemolytic mastitis strains, and hemolytic human strains, showed considerable variation in the production of amino compounds by the different members of the various groups. In a general way, it may be said that the increases in amino nitrogen exhibited by the lactic strains studied, cover about the same range as those exhibited by the hemolytic mastitis strains. The range of amino acid production by these types is usually less than the amount produced by the representative of L. C. Avery's hemolytic cheese group. The above statements are, of course, conditioned by the number of strains studied and are limited entirely to them and to the conditions of the present experiment.

This divergence in amino acid production in peptone broth is probably of no diagnostic importance. It is, however, of peculiar interest from the standpoint of microbial physiology. Its economic importance is probably limited to its contribution to a more intimate knowledge of the metabolism of streptococci which are important in agriculture and public health.

The explanation of the apparent divergence in the extent of peptone hydrolysis, is possible only after more intimate and extended studies of the various factors and forces which are involved in the formation of amino compounds by microorganisms. Some of these have already been suggested in Section A. Among

the most important of those now recognized are the potential activity of the enzymes involved, the concentration of the enzymes, the liberation of the enzymes into the hydrolysis system itself, the stability of the enzyme activity in certain environments, and the availability of the substrate. Other forces unrecognized at the present time are probably also involved. Moreover, the relative moment of all of these forces is conditioned, and possibly to a different degree, by all of the conditions in the environment.

The question of differences in the potential activity of the enzymes of the different types of streptococci is indeed an interesting and suggestive possibility. Such questions have repeatedly been raised in connection with the relative virulence of different, but closely related microorganisms. Their solution, of course, requires the isolation of unaltered and not inactivated enzymes which is not always possible. It is always possible that there is little difference in the potential activity of similar enzymes of such closely related organisms,- and that quantitative differences observed in their products is due to the limitation of their operation by one or more of the above mentioned conditions. In consideration of the results of Avery and Cullen, the relation of virulence to the activity of the proteolytic enzymes of pneumococci would seem to be remote.

The concentration of the enzymes is closely related, but not necessarily parallel to the growth and number of the cells. It is interesting to observe that many of the human strains which give apparently delicate growth in broth culture, give much higher increases in amino nitrogen than do the seemingly more luxuriantly

growing lactic and bovine strains.

The relation of disintegration of cells and the consequent liberation of endo enzymes is also interesting, especially in consideration of the results obtained in experiment 4 (Section A) with the human and lactic strains.

Increases in Ammonia Nitrogen.

The production of ammonia was found to vary to a certain extent within the lactic group. The different types of streptococci did not exhibit any marked difference in ammonia nitrogen increases in the media used. There seemed to be no definite relation between the increases in amino and in ammonia nitrogen, - neither between the different members of the lactic group nor between the strains representative of the other types.

These relations suggest that the production of ammonia is largely due to the operation of different processes than is the production of amino compounds. It was indicated in Section A that the formation of ammonia in peptone broth cultures is associated more strictly with the actual growth and life of streptococci than is that of amino acids.

GENERAL SUMMARY.

Section A.

The relative influence of environmental conditions was investigated for the purpose of establishing the optimum conditions for the peptolytic action of different types of streptococci. As an index of the successful operation of the peptolytic processes, measurements were made of the relative amounts of amino and ammonia nitrogen which were formed by the different streptococci when growing in different environments.

The results of this section of the study were used as a means of standardizing the systems to be used for the investigation in Section B. of the peptolytic action of a number of strains of the different types.

In addition to the standardization of the optimum conditions for the investigation pursued in Section B, the results of this study yielded the following summarized facts of independent interest and importance.

1. The production of amino compounds in broth cultures of streptococci seems to continue beyond the period of growth of the culture. Significant increases occur, especially with the human strain, after the cessation of growth and during the period of death of the cells.

2. A greater portion of the total increase in ammonia occurs earlier in the history of the culture than in the case of the amino acid increases. This may indicate that ammonia production is associated more strictly with the growth and active life of

streptococci than is the accumulation of amino compounds.

3. The human and bovine strains exhibited an optimum H-ion concentration zone of pH 7.0 to 8.0. The lactic and cheese strains seem to prefer a zone of pH 6.0 to 7.0. The optimum pH zone for growth of the cells agreed with the zone in which occurred the greatest amino nitrogen increase for the respective strains.

4. The ability of the cheese strain to split peptone is inhibited to a less extent by H-ion concentrations approximating pH 5.5 than is that of any of the other type strains.

5. In broth of pH 4.5, the type strains exhibit the following order of acid-tolerance: cheese, lactic, bovine, and human. The cheese strain exhibits a greater relative resistance to the disinfectant action of this concentration of the H-ion than would seem to follow from a comparison of the "fermentation limits" or final H-ion concentrations exhibited in glucose broth by the bovine and lactic strains.

6. Further examples are furnished that show that the "limiting initial H-concentrations" for different types of streptococci in plain broth, do not coincide with their "fermentation limits" in glucose broth.

7. The rate of growth of the lactic streptococcus is retarded to a less extent by low temperatures than is that of the hemolytic type strains. The same is also true of its rate of amino acid production.

8. Examples are furnished which show that higher final increases in amino nitrogen may occur at temperatures lower than that at which the greatest rate of amino acid production takes place.

9. Temperatures somewhat above the optimum may effect the final H-ion concentration of glucose broth cultures of streptococci.

10. The life processes of the cheese and bovine strains are inhibited to a less extent by a temperature of 41° than are those of the lactic and human strains. This is evident by the rate of growth, the activity of peptone attack, the final H-ion concentration, and the longevity of cultures of the different strains, when incubated at this temperature.

11. At a temperature of 41° the final amino nitrogen increases by the hemolytic human streptococcus were conditioned by the size of the inoculum. At 37°, the same limits of size of inoculum were apparently without effect upon the total production of amino acids.

12. With the exception of the cheese strain, all of the type strains proved able to attack peptone as successfully under low oxygen concentrations as under aerobic conditions.

13. The study of the influence of environmental conditions upon the different types of streptococci presents results which indicate that the lactic and cheese types are better fitted to survive the conditions extant in fresh milk, and in milk and milk products at later periods of its handling.

14. The cheese strain, by reason of its striking resistance to high acidities and to long exposures to unfavorable environments, is peculiarly adapted to the struggle for existence in the microbial balance of milk products. These relations may explain its appearance in comparatively large numbers in certain dairy products, even though it were initially present in relatively small numbers.

Section B.

Section B is concerned with measurements of the amino and ammonia compounds which are formed by a larger number of strains of different types of streptococci when growing in peptone broth under the environmental conditions established as optimum for the different streptococci in Section A. The peptolytic action of the lactic and cheese strains were studied at 31° C. in a pH zone approximately that of fresh milk; the peptolytic action of the hemolytic human and bovine strains were studied at 37° C. in a pH zone approximately that of blood and of the usual bacteriological media.

The results obtained may be summarized as follows.

15. Broth cultures of non-hemolytic lactic, hemolytic human, and hemolytic mastitis streptococci showed considerable variation in the increases in amino and ammonia nitrogen effected by different strains of the same type.

16. The increases in amino nitrogen exhibited by the eight hemolytic mastitis strains in broth of initial pH 7.8, covered approximately the same range as that shown by seventeen non-hemolytic sour milk or lactic strains in broth of initial pH 6.6.

17. Eleven hemolytic human strains produced larger increases in amino nitrogen than did the hemolytic mastitis and non-hemolytic lactic strains.

18. With certain streptococci, increases in amino nitrogen may not be evident until after an extended period of incubation.

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